REMARKS

Claims 7, 9, 10, 13-15, and 23 were pending as of the December 20, 2002 Official Action. While not necessarily in agreement with the rejections made by the Examiner, Applicants have amended the claims to expedite review and allowance. Applicants also reserve the right to prosecute any cancelled claim matter in later applications.

The Examiner's grounds for rejecting all application claims is traversed as set forth below.

I. THE SECTION 112, 2nd PARAGRAPH REJECTION OF CLAIM 27

The Examiner rejected claim 27 as being indefinite because it is the Examiner's position that the identification of the persons in need of treatment is uncertain and indefinite. This objection has been overcome by (1) amending the claim to make it definite; and (2) by submitting a Declaration by one of the inventors – Dr. Italo Biaggioni.

Claim 27 is amended above to direct the method to the treatment in mammals of a "disease, disorder or condition mediated by antagonizing A2B receptors. . . " This claim amendment succinctly identifies the group of mammals covered by the claim.

The Examiner also takes the position that the claim is indefinite because relatively little is known about the effects of the receptor, and therefore, there is no way of knowing just who this claim covers. A Declaration of Dr. Italo Biaggioni, one of the application inventors, is attached to this Reply at Appendix A. In his Declaration, Dr. Biaggioni identifies and includes copies of many articles that discuss the many diseases, disorders and conditions mediated by antagonizing the A2B receptor. (See Biaggioni Dec. ¶¶6-8). Dr. Biaggioni's Declaration concludes that one of ordinary skill in the art at the time of the invention would be aware of the diseases, disorders and conditions mediated by antagonizing the A2B receptor. (See Biaggioni Dec. ¶9). Dr. Biaggioni's Declaration, along with the claim 27 amendments presented above are believed, therefore, to overcome the Examiner's rejection of claim 27 for indefiniteness.

II. THE DOUBLE PATENTING REJECTION

The Examiner provisionally rejected claims 7, 9-10, 13-15, 23, 27 and 28 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims

of co-pending and related application serial no. 10/285747.

This rejection is overcome by filing a Terminal Disclaimer related to application no. 10/285747 contemporaneously with the filing of this Reply. A copy of the Terminal Disclaimer is attached to this Reply at Appendix B.

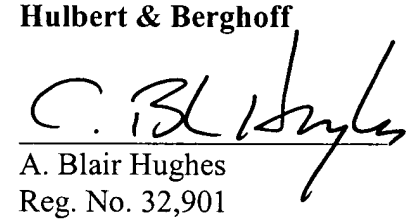
CONCLUSION

Applicants request the Examiner to reconsider the rejections in view of the above arguments and claim amendments. Favorable reconsideration and allowance of the pending application claims is therefore courteously solicited.

Respectfully submitted,

McDonnell Boehnen Hulbert & Berghoff

Dated: November 19, 2003

By: 

A. Blair Hughes
Reg. No. 32,901
312-913-2123

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Case No. MBHB00-618-A)

In the Application of:	)	
	)	
Italo O. Biaggioni et al.	)	
	)	Art Unit: 1624
Serial No.: 09/648,775	)	
	)	Examiner: M. Berch
Filed: August 28, 2000	)	
	)	
Title: Selective Antagonits of A2B Adenosine Receptors	)	

Commissioner for Patents
Washington, D.C. 20231

Sir:

DECLARATION OF DR. ITALO BIAGGIONI
(PURSUANT TO 37 C.F.R SECTION 1.132)

I Italo Biaggioni, residing at 2873 Sugartree Rd, Nashville, TN 37215, do hereby declare:

1. I am a named co-inventor of this United States Letters Patent Application Serial No. 09/648,775, filed on August 28, 2000.

2. I hold a M.D degree in Medicine from Universidad Peruana Cayetano Heredia, Lima, Peru.

3. I am currently, Professor of Medicine and Pharmacology at Vanderbilt University School of Medicine. I have been actively pursuing the design of drugs and Pharmacological research at Vanderbilt University school of Medicine for 20 years. I have been actively engaged in the study of Adenosine A2B receptors and human diseases and conditions treatable with antagonists of Adenosine A2B receptors for 8 years. Prior to joining the Faculty at Vanderbilt, I did three years training in internal medicine in Lima, Peru and three years of fellowship training in Clinical Pharmacology at Vanderbilt University.

4. I have reviewed the specification of the above-captioned patent application.

5. One aspect of the invention claimed in the above-captioned patent application is a method for treating diseases, disorders or conditions mediated by the A2B receptors in a

mammal such as a human by administering to a mammal in need of such mediation a therapeutically effective amount of a claimed A2B antagonist compound.

6. Those persons working in the area of A2B receptors are aware of mammalian diseases, disorders, and conditions that are mediated by the A2B receptor. The pertinent diseases, disorders, and conditions are described in various textbooks and articles related to Adenosine receptors.

7. I have attached copies of each of the following references to this Declaration:

- Auchampach JA, Jin X, Wan TC, Caughey GH, and Linden J (1997) Canine mast cell adenosine receptors: Cloning and expression of the A_3 receptors and evidence that degranulation is mediated by the A_{2B} receptor. *Mol.Pharmacol.* **52**:846-860. (Tab A).

- Feoktistov I and Biaggioni I (1995) Adenosine A2b receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. *J.Clin.Invest.* **96**:1979-1986. (Tab B).

- Feoktistov I and Biaggioni I (1997) Adenosine A_{2B} receptors. *Pharmacol.Rev.* **49**:381-402. (Tab C).

- Feoktistov I, Polosa R, Holgate ST, and Biaggioni I (1998a) Adenosine A_{2B} receptors - a novel therapeutic target in asthma? *Trends Pharmacol.Sci.* **19**:148-153. (Tab D).

- Feoktistov I, Wells JN, and Biaggioni I (1998b) Adenosine A_{2B} receptors as therapeutic targets. *Drug Dev.Res.* **45**:198-206. (Tab E).

- Grant MB, Tarnuzzer RW, Caballero S, Ozeck MJ, Davis MI, Spoerri PE, Feoktistov I, Biaggioni I, Shryock JC, and Belardinelli L (1999) Adenosine receptor activation induces vascular endothelial growth factor in human retinal endothelial cells. *Circ.Res.* **85**:699-706. (Tab F).

- Strohmeier GR, Reppert SM, Lencer WI, and Madara JL (1995) The A2b adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. *J.Biol.Chem.* **270**:2387-2394. (Tab G).

I am listed as co-author of the references attached at Tabs B, C, D & E. I have reviewed and am familiar with each of the remaining references.

8. The references teach the following about diseases, disorders, and conditions that

are mediated by the A2B receptor:

A. Mast cell activation, asthma, and other inflammatory processes

Adenosine is known to activate mast cells that express A_{2B} receptors. This has been shown in human (Feoktistov and Biaggioni, 1995) and canine (Auchampach et al., 1997) mast cells. Mast cells are involved in allergies, asthma, and other inflammatory processes. Adenosine, given by inhalation as its precursor AMP, is known to provoke asthma in asthmatics by activation of mast cells (for review see (Feoktistov et al., 1998a). Inhibition of mast cell activation using A_{2B} antagonists, therefore, may be useful in the treatment of asthma, allergies, and other inflammatory processes.

B. Angiogenesis

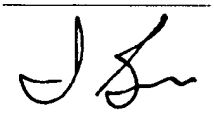
Adenosine A_{2B} receptors are present in endothelial cells, and their activation mediates endothelial cells growth (Grant et al., 1999), an important step for the formation of new blood vessels ("angiogenesis"). A_{2B} antagonists, therefore, may be useful in conditions characterized by abnormal blood vessel growth, as occurs in diabetic retinopathy,

C. Intestinal secretion

Activation of adenosine A_{2B} receptors present in intestinal epithelial cells stimulates chloride secretion (Strohmeier et al., 1995). This is thought to be the mechanism my which neutrophils induce diarrhea. A_{2B} antagonists, therefore, may be useful in the treatment of diarrhea induced by inflammatory or infectious processes.

9. In view of the above points, the diseases, disorders and conditions treatable by A2B receptor antagonists are well known and those working in this area would be aware of such diseases, disorders and conditions. Therefore, those working in this area would understand which humans are in need of treatment with A2B receptor antagonist compounds of our invention.

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that theses statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: 

Dr. Italo Biaggioni

Date: November 19, 2003

0026-895X/97/050846-15$3.00/0

MOLECULAR PHARMACOLOGY, 52:846–860 (1997).

Canine Mast Cell Adenosine Receptors: Cloning and Expression of the A_3 Receptor and Evidence that Degranulation Is Mediated by the A_{2B} Receptor

JOHN A. AUCHAMPACH, XIAOWEI JIN, TINA C. WAN, GEORGE H. CAUGHEY, and JOEL LINDEN

Departments of Medicine (Cardiology), and Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908 (J.A.A., X.J., T.C.W., J.L.), and Department of Medicine, Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143 (G.H.C.)

Received June 17, 1997; Accepted July 31, 1997

SUMMARY

We cloned and characterized the canine A_3 adenosine receptor (AR) and examined AR-induced degranulation of the BR line of canine mastocytoma cells. Canine A_3AR transcript is found predominantly in spleen, lung, liver, and testes and encodes a 314-amino acid heptahelical receptor. ^{125}I-N^6-Aminobenzyladenosine binds to two affinity states of canine A_3AR with K_D values of 0.7 ± 0.1 and 16 ± 0.8 nM, reflecting G protein-coupled and -uncoupled receptors, respectively. Xanthine antagonists bind with similar affinities to human, canine, and rabbit receptors but with 80–400-fold lower affinities to rat A_3AR. Although canine BR mastocytoma cells contain A_1AR, A_{2B}AR, and A_3AR, degranulation seems to be mediated primarily by A_{2B}ARs stimulated by the nonselective agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) but not by the A_3-selective agonist N^6-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide. NECA-stimulated degranulation is not prevented by pertussis toxin and is blocked by enprofylline (K_i = 7 μM), an antiasthmatic xanthine with low affinity (K_i > 100 μM) for A_1AR, A_{2A}AR, and A_3AR. NECA increases canine mastocytoma cell cAMP, Ca^{2+}, and inositol trisphosphate levels; these responses are antagonized half-maximally by 7–15 μM enprofylline. The results suggest that (i) the cloned canine A_3AR is structurally and pharmacologically more similar to human than to rat A_3AR; (ii) the A_{2B}AR, and not the A_1AR or A_3AR, is principally responsible for adenosine-mediated degranulation of canine BR mastocytoma cells; and (iii) the BR cell A_{2B}AR couples to both Ca^{2+} mobilization and cAMP accumulation. Although A_{2B} receptors play a major role in the regulation of BR mast cell degranulation, multiple AR subtypes and G proteins may influence mast cell functions.

Adenosine exerts numerous physiological effects that were originally thought to be mediated by three adenosine receptors, A_1, A_{2A}, and A_{2B}. In the early 1990s, a new adenosine receptor was cloned from rat tissues, first by Meyerhof *et al.* (1) and then by Zhou *et al.* (2), who named it A_3. More recently, human (3), sheep (4), and rabbit (5) A_3 adenosine receptors have been cloned and characterized. Functional expression of A_3 adenosine receptors from various species indicates that A_1 and A_3 receptors bind the radioligands [^{125}I]APNEA, [^{125}I]ABA, and [^{125}I]AB-MECA and are negatively coupled to adenylyl cyclase (2, 4, 6). One unusual property of A_3 adenosine receptors is a major difference among species in the binding affinity of xanthine antagonists. In particular, the rat receptor is resistant to blockade by xanthines, whereas sheep, human, and rabbit receptors bind certain xanthines with high affinity, although with distinct potency orders (3).

The addition of adenosine to rat basophilic leukemic cells (RBL 2H3 cells; a tumor cell line resembling mast cells) causes facilitation of the release of granules, which is mediated by A_3 adenosine receptors (7, 8). A_3 receptor activation also triggers the degranulation of mast cells surrounding hamster cheek pouch arterioles (9). Based on these results, the observation that the inhalation of adenosine produces histamine release and bronchoconstriction in asthmatics but

This work was supported by National Institutes of Health Grants RO1-HL37942 and T32-HL07284.

ABBREVIATIONS: [^{125}I]APNEA, N^6-2-(4-amino-3-[^{125}I]iodophenyl)adenosine; [^{125}I]ABA, N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine; [^{125}I]AB-MECA, N^6-(4-amino-3-[^{125}I]iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; XAC, 8-(4-[(2-aminoethyl)aminocarbonylmethyloxy]-phenyl)-1,3-dipropylxanthine; CPA, N^6-cyclopentyladenosine; AM, acetoxymethyl ester; (*R*)-PIA, (*R*)-N^6-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; I-ABOPX, 3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine; 8-SPT, 8-sulfophenyltheophylline; NBTI, nitrobenzylthioinosine; RT, reverse transcription; PCR, polymerase chain reaction; BSA, bovine serum albumin; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; $InsP_3$, inositol trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;.

not in nonasthmatics (10, 11) and the discovery of high levels of A_3 adenosine receptor transcript in human and sheep lung, we proposed a role for the A_3 adenosine receptor in the pathophysiology of asthma (12). Because rodents may be poor animal models for the investigation of the role of A_3 receptors in human allergy and asthma, we decided to clone the canine A_3 adenosine receptor as a first step toward characterizing the role of A_3 adenosine receptors in canine models of asthma.

Here, we report the cloning, expression, and pharmacological characterization of an A_3 adenosine receptor cDNA isolated from BR cells [canine mastocytoma cells (13)]. Low levels of both A_1 and A_3 adenosine receptors are found on BR cells, but these are not primarily responsible for stimulating degranulation of this canine mastocytoma cell line. Rather, an A_{2B} adenosine receptor causes degranulation via a pertussis toxin-insensitive pathway that mobilizes mastocytoma cell Ca^{2+} and can be blocked by the antiasthmatic xanthine enprofylline (14).

Experimental Procedures

Materials. All chemicals were obtained from Sigma Chemical (St. Louis, MO) with the following exceptions. IB-MECA was from Dr. Saul Kadin (Pfizer, Groton, CT). I-ABA and I-ABOPX (also known as BW-A522) were from Dr. Susan Daluge (Glaxo-Wellcome, Research Triangle Park, NC). WRC-0571 [C^8-(*N*-methylisopropyl)-amino-N^6-(5′-endohydroxy)-endonorbornan-2-yl-9-methyladenine] was from Dr. Pauline Martin (Discovery Therapeutics, Richmond, VA). APNEA was from Dr. Ray Olsson (University of South Florida, Tampa, FL). RDC7 (dog A_1 adenosine receptor cDNA) was from Dr. Guy Vassart (Brussels, Belgium). Rat A_3 adenosine receptor cDNA was from Dr. Fereydoun Sajjadi (Gensia, La Jolla, CA). Human A_3 adenosine receptor cDNA was from Dr. Marlene Jacobson (Merck, West Point, PA). Rabbit A_3 adenosine receptor cDNA was from Dr. Scott Kennedy (Pfizer, Groton, CT). HMC-1 mast cells were from Dr. J. H. Butterfiled (Mayo Clinic, Rochester, MN). NECA, CGS 21680 (2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine), (*R*)-PIA, CPA, CPX, XAC, 8-SPT, theophylline, and enprofylline were purchased from Research Biochemicals (Natick, MA). Ro 20–1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Adenosine deaminase was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Fura-2/AM was from Molecular Probes (Eugene, OR). *myo*-[^{3}H]Inositol was from Amersham Life Sciences (Arlington Heights, IL). Dowex AG 1- X8 was from BioRad (Richmond, CA). [^{125}I]ABA was synthesized as described previously (15). Cell culture media and supplies were from GIBCO BRL (Gaithersburg, MD).

Cell culture. COS-7 cells were grown in DMEM with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Canine BR mastocytoma cells were grown in low-glucose DMEM supplemented with 2% calf serum, 25 mM HEPES, 1.5 mM *l*-histidine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. The medium was changed every 3 days, and the cells were replated weekly.

Molecular cloning. To obtain the full-length sequence of the canine A_3 adenosine receptor cDNA, a library prepared in λgt10 from BR cell poly(A)$^+$ RNA was screened using a probe generated by RT-PCR of total RNA isolated from dog tissues using the cDNA cycle kit (InVitrogen, La Jolla, CA). Primers for amplification were primer A (sense 132–152), 5′-GACCACCACCTTCTATTTCA-3′; and primer B (antisense 660–680), 5′-GTCTTGAACTCCCGA/TCC-3′. The primers correspond to conserved regions within the first and third intracellular loops of the human, sheep, and rat A_3 adenosine receptor cDNAs. Each reaction cycle consisted of incubations at 95° for 1 min, 55° for 2 min, and 72° for 3 min with 0.02 unit/ml of *Taq* polymerase (Promega, Madison, WI). PCR fragments were subcloned into the TA vector (InVitrogen) and sequenced with Sequenase (United States Biochemical, Cleveland, OH) using modifications for double-stranded sequencing. One fragment from lung RNA was found to be ~90% identical to the human, rat, and sheep A_3 receptor transcripts. This probe was labeled with [α-^{32}P]dCTP (Primit II; Stratagene, La Jolla, CA) and used to screen the BR cell cDNA library. Library screening was carried out by plaque-filter hybridization. Filters were hybridized at 65° overnight in 10% dextran sulfate, 1 M NaCl, 100 μg/ml herring sperm DNA, and 1×10^6 cpm/ml radiolabeled probe and then washed in 0.5× SSC/0.5% SDS at 65°. Recombinants hybridizing to the probe were plaque-purified and reprobed. Recombinant phage DNA isolated by the plaque lysate method were digested with *Eco*RI and electrophoresed through a 1% agarose gel to determine the insert sizes. Several clones were identified ranging in size from 0.9 to 3 kb. One clone ($cA_3$13.1), which was 1.6 kb long, was subcloned into the *Eco*RI site of the plasmid vector pGEM-7z(−) (Promega). Double-stranded DNA was isolated, and both strands were sequenced in full, first by using T7 and Sp6 primers to get nucleotide sequence information near the 5′ termini, and then with a series of synthetic oligonucleotide primers derived from sequences determined previously.

Radioligand binding studies. Membranes were prepared from COS-7 cells expressing the canine A_3 receptor ($cA_3$13.1) or the canine A_1 receptor (RDC7); HEK 293 cells stably expressing human, rabbit or rat A_3 adenosine receptors; or canine BR cells. The full coding region of the receptor cDNAs were subcloned into the expression vector CLDN10B and transiently expressed (60 hr) in COS cells by the DEAE-dextran method (16) or stably expressed in HEK 293 cells after transfection by the Ca^{2+} phosphate precipitation method (17) and selection in 2 mg/ml G-418. Transfected cells were washed in phosphate-buffered saline; homogenized in 10 mM EDTA, 10 mM Na-HEPES, pH 7.4, and 0.1 mM benzamidine; and centrifuged at 20,000 × *g* for 20 min. Pellets were resuspended and washed in 10 mM Na-HEPES, 1 mM EDTA, pH 7.4, and 0.1 mM benzamidine (HE buffer) and resuspended in the same buffer with 10% (w/v) sucrose (sucrose buffer) at a membrane protein concentration of 1 mg/ml. Because [^{125}I]ABA bound poorly to crude BR cell membranes, plasma membranes were enriched by preparing P2 pellets. Cells were homogenized in sucrose buffer and centrifuged at 500 × *g* for 10 min. The pellet was resuspended in sucrose buffer and centrifuged again at 500 × *g*. The pooled supernatants were diluted 3-fold, pelleted, and washed twice by centrifugation at 20,000 × *g* for 20 min in HE buffer; resuspended; and frozen in sucrose buffer. Protein concentrations were determined using fluorescamine with BSA as standard. Membranes were frozen in aliquots and stored at −80°. For radioligand binding studies, cell membranes were incubated in 0.1 ml for 3 hr at 21° with 5 mM $MgCl_2$ and 5 units/ml adenosine deaminase. For equilibrium binding assays, 6–8 concentrations of [^{125}I]ABA were used in triplicate in tubes, each containing 10–60 μg of membrane protein, and the specific activity of [^{125}I]ABA was reduced 10–20-fold with the nonradioactive compound. Nonspecific binding was measured in the presence of 5 μM I-ABA. [^{125}I]ABA was found to have a higher ratio of specific to nonspecific binding than an alternative radioligand, [^{125}I]AB-MECA. For competition experiments, 0.5–1 nM [^{125}I]ABA was added to tubes, and competing ligands were added over a range of concentrations; the tubes contained 10–50 μg of membrane protein in a final volume of 0.1 ml.

Analysis of binding data. Specific [^{125}I]ABA binding to A_1 adenosine receptors was optimally fit to a single site binding model using Marquardt's nonlinear least-squares interpolation (18). [^{125}I]ABA was found to bind to two affinity states of the recombinant canine, human, and rat A_3 receptors. For two-site Scatchard transformation, the relationship between bound/free and bound can be shown to be described by a quadratic equation: bound/free = $A * X * X + B * X + C$, where A = bound; $X = K_{d1}/K_{d2}$; $B = K_{d1} * X + K_{d2} * X - B_{max1} * K_{d2} - B_{max2} * K_{d1}$; $C = X * X - B_{max1} * X - B_{max2} * X$. Optimal parameters for two-site Scatchard plots were generated by using the

binomial theorem to solve this equation within each iteration of nonlinear least-squares analysis.

IC_{50} values of compounds in competition experiments were fit to $SB_i = B_i - (B_i - NS)\,[I]/(IC_{50i} + [I])$ where i is the number of binding sites, SB is specific binding, and NS is nonspecific binding. K_i values were calculated from IC_{50}, B_{max}, the concentration of [^{125}I]ABA, and its K_d value, as described previously (19). For A_3 receptors, the determination of the K_i values of competing agonists for an agonist radioligand ([^{125}I]ABA), is complicated by the fact that both the radioligand the competing compounds bind to two affinity states. This is described by four equations: $LB = B_{max1} * (L/K_{d1})/(1 + L/K_{d1} + C/K_{i1}) + B_{max2} * (L/K_{d2})/(1 + L/K_{d2} + C/K_{i2}) + f * L$; $CB = B_{max1} * (C/K_{i1})/(1 + L/K_{d1} + C/K_{i1}) + B_{max2} * (C/K_{i2})/(1 + L/K_{d2} + C/K_{i2}) + f * C$; $LT = L + LB$; $CT = C + CB$; where LB is radioligand bound, CB is competitor bound, L is free radioligand, C is free competitor, and f is fraction of L or C nonspecifically bound (assumed to be equal). K_{d1}, K_{d2}, and the fraction of coupled receptors were derived from equilibrium radioligand binding in the absence of competitor. The other parameters were determined by simultaneously solving these four equations by interpolation within each iteration of nonlinear least-squares analysis. For the analysis of antagonist binding, K_{i1} and K_{i2} values were set to be equal based on the assumption that antagonists bind with similar affinities to G protein-coupled and -uncoupled receptors.

Northern blots. Northern analysis and RT-PCR were used to determine the tissue distribution of A_3 adenosine receptor transcript and to identify A_{2B}, A_1, and A_3 receptor transcripts in BR cells. Total RNA was extracted and poly(A)$^+$ RNA was selected using oligo(dt) cellulose. Five micrograms of poly(A)$^+$ RNA was electrophoresed through 1% agarose gels containing 1% formaldehyde and then transferred to nylon membranes (Genescreen Plus; DuPont). The membranes were hybridized in 10% dextran sulfate, 1 M NaCl, and 100 μg/ml herring sperm DNA with 1×10^6 cpm/ml random-labeled probe at 65° overnight. Filters were washed with 0.5× SSC/0.5% SDS at 65° and then exposed to Amersham Hyperfilm MP for 24–48 hr. The A_3 receptor probe consisted of a 600-bp PCR fragment of $cA_3$13.1, corresponding to approximately half of the carboxyl-terminal sequence and the 3′ noncoding region. The A_{2B} receptor probe consisted of a 500-bp PCR fragment generated by RT-PCR from BR cell RNA corresponding to transmembrane regions I–IV.

For RT-PCR, 1 μg of poly(A)$^+$-selected RNA was reverse-transcribed and amplified using primers A and B described above. The reactions were electrophoresed and transferred to nylon membranes (Hybond N$^+$; Amersham) using denaturing buffer (0.4 N NaOH) and hybridized with specific probe. Filters were washed under stringent conditions (0.1× SSC/0.5% SDS at 65° for 1 hr). Control reactions were included in which RT or RNA was excluded from the reactions. Some of the PCR fragments were subcloned into the TA vector and partially sequenced.

Mastocytoma cell degranulation. As an indicator of degranulation of BR cells, we measured the release of β-hexosaminidase (a granule-associated protein that parallels histamine release) using a modification of the method of Schwartz *et al.* (20). BR cells grown in suspension were washed twice in Ca^{2+}/Mg^{2+}-free Tyrode's buffer and then resuspended in complete Tyrode's at a density of 1.2×10^6 cells/ml. Cells were then transferred to a 96-well plate in 250-μl aliquots and prewarmed to 37° for 15 min. Cells were stimulated with agonists added in 50-μl aliquots for 20 min at 37° with shaking. The reactions were stopped by placing the plate on ice for 10 min and then pelleting the cells by centrifugation at $200 \times g$ for 10 min (4°). Two hundred microliters of the supernatant was removed and added to 50 μl of 5 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide, and 100 mM citric acid, pH 3.8, and incubated at 37° for 2 hr with shaking before the addition of 50 μl of 0.4 M $NaCO_3$. Total cellular β-hexosaminidase was determined by adding 50 μl of lysis buffer (complete Tyrode's buffer plus 0.6% Triton A-100) to 250-μl aliquots of cells, and 20 μl was removed and assayed. Absorbance was read at 405 nm using a Titertech Multiskan II plate reader. Experiments were performed in triplicate, and release of β-hexosaminidase is expressed as percentage of the total content of unstimulated cells.

cAMP. BR cells were washed twice and resuspended in serum-free low-glucose DMEM containing 25 mM HEPES, 1 unit/ml adenosine deaminase, and 20 μM Ro 20–1724 and then transferred to polypropylene test tubes (1×10^6 cells/0.2 ml, 21°). Drugs were added in 50-μl aliquots, and the tubes were placed in a 37° shaking water bath for 20 min. Assays were terminated by the addition of 500 μl of 0.15 N HCl. cAMP in the acid extract (500 μl) was acetylated and quantified by automated radioimmunoassay.

Intracellular Ca^{2+}. BR cells were loaded with 1 μM Fura-2/AM in buffer containing 100 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 2.7 g/liter D-glucose, 20 mM Na-HEPES, pH 7.4, and 0.25% BSA for 45 min. Cells were washed and resuspended in the same buffer without BSA, plus 1 unit/ml adenosine deaminase to a density of 1×10^6 cells/ml. Fluorescence was measured with an SLM spectrofluorimeter in a thermostable cuvette (37°).

$InsP_3$. BR cells were preincubated for 24 hr with 2.5 μCi/ml *myo*-[^{3}H]inositol in inositol-free low-glucose DMEM supplemented with 2% dialyzed fetal calf serum. The labeled cells were washed and resuspended in low-glucose DMEM with 25 mM HEPES, 1 unit/ml adenosine deaminase, and 100 mM LiCl and then transferred to polypropylene test tubes (4×10^5 cells/0.2 ml) at 37° in a shaking water bath and stimulated by 5× agonists added in 50-μl aliquots for 10 min. Assays were terminated by the addition of 400 μl stop solution (0.5 M $HClO_4$, 5 mM EDTA, and 1 mM diethylenetriaminpentacetic acid) plus 1 mg/ml phytic acid and placed on ice for 30 min before the addition of 5 M K_2CO_3 to raise the pH to 8–9. After centrifugation, the supernatants were passed through a 0.2-μm filter, applied to 1-ml Dowex AG 1-X8 columns (200–400 mesh), and washed with 5 ml of H_2O and 5 ml of 40 mM HCl; then, $InsP_3$ was eluted with 5 ml of 170 mM HCl.

Results

Molecular cloning of the canine A_3 adenosine receptor. The screening of a canine mastocytoma cDNA library with an A_3 adenosine receptor probe generated by RT-PCR resulted in the identification of several positively hybridizing clones. A clone designated $cA_3$13.1 contains a 1.6-kb insert with an open reading frame corresponding to 314 amino acids and 181 and 480 bp of 5′ and 3′ untranslated sequence, respectively (Fig. 1). A hydrophilicity plot of the deduced amino acid sequence predicts seven transmembrane domains, which are indicated in Fig. 2A. Sites found to be conserved within all species of A_3 adenosine receptors cloned to date include a putative palmitoylation site at Cys305 of the consensus sequence and two putative *N*-linked glycosylation sites at Asn4 and Asn162. Several putative phosphorylation sites are conserved among the A_3 adenosine receptors, including four potential sites for phosphorylation by protein kinase C (Thr124, Thr125, Ser/Thr215, and Thr230); one potential site for phosphorylation by tyrosine kinases (Tyr120); and one potential site for phosphorylation by cAMP/cGMP-dependent protein kinases (Thr294). The carboxyl tail distal to the palmitoylation site contains several serine/threonine residues that are surrounded by acidic groups that may be sites for phosphorylation by G protein receptor kinases.

The deduced amino acid sequence of $cA_3$13.1 is 88%, 86%, 72%, and 77% identical to the human, sheep, rat, and rabbit receptors (Fig. 2B), respectively, and 52% and 47% identical to canine A_1 and A_{2A} receptors, suggesting that $cA_3$13.1 is the canine species homolog of the A_3 adenosine receptor. Be-

```
1     cgggaggctctccaagggagcgtcccaccagagaagagaaggaatgagcaagttgtgaat   60
61    ttgggaccgttgctgttgcacctgaacctctagcgagatgcctggcaagagctaggctca  120
121   ccgggccacacggatcctgtcagcacgcctattacttgggaagctccttggagaaagcaa  180

          M  A  V  N  G  T  A  L  L  L  A  N  V  T  Y  I  T  V  E  I
181   gatggctgtcaatggcactgccctgttgttggccaatgtcacctacatcacagtggagat  240
                                    10
          L  I  G  L  C  A  I  V  G  N  V  L  V  I  W  V  V  K  L  N
241   tctcatcgggctctgcgccatcgtgggcaatgtgttggtcatctgggtggtcaagctgaa  300
                                    30
          P  S  L  Q  T  T  T  F  Y  F  I  V  S  L  A  L  A  D  I  A
301   ccccagcctacagaccaccaccttctatttcattgtctccctggcccttgctgacattgc  360
                                    50
          V  G  V  L  V  M  P  L  A  I  V  I  S  L  G  I  T  I  Q  F
361   cgttggggtgctggtcatgcctttggccattgtcatcagcctgggcatcacaatccaatt  420
                                    70
          Y  N  C  L  F  M  T  C  L  L  L  I  F  T  H  A  S  I  M  S
421   ttataactgccttttcatgacctgcctgctgttgatcttcactcatgcttccatcatgtc  480
                                    90
          L  L  A  I  A  V  D  R  Y  L  R  V  K  L  T  V  R  Y  R  R
481   cctgctagccattgctgtggaccggtacctgcgggtcaagctcacagtcagatacaggag  540
                                   110
          V  T  T  Q  R  R  I  W  L  A  L  G  L  C  W  L  V  S  F  L
541   ggtcaccacacaaagaagaatatggttggccctggggctttgctggctggtgtccttcct  600
                                   130
          V  G  L  T  P  M  F  G  W  N  M  K  L  T  S  E  H  Q  R  N
601   ggtgggactgacccccatgtttggctggaacatgaaactgacctcagagcaccagagaaa  660
                                   150
          V  T  F  L  S  C  Q  F  S  S  V  M  R  M  D  Y  M  V  Y  F
661   tgtcaccttcctctcgtgccagttcagttctgtcatgaggatggactacatggtctactt  720
                                   170
          S  F  F  T  W  I  L  I  P  L  V  V  M  C  A  I  Y  L  D  I
721   cagcttcttcacttggatcttaatccccctggttgtcatgtgtgccatctatcttgacat  780
                                   190
          F  Y  V  I  R  N  K  L  N  Q  N  F  S  S  S  K  E  T  G  A
781   attctatgtcatccggaacaaactcaatcagaacttttcaagctccaaagagacaggtgc  840
                                   210
          F  Y  G  R  E  F  K  T  A  K  S  L  F  L  V  L  F  L  F  A
841   attttatggacgggagttcaagacggccaagtccctgtttctggttctcttcctgtttgc  900
                                   230
          F  S  W  L  P  L  S  I  I  N  C  I  T  Y  F  H  G  E  V  P
901   tttttcctggctgcctttatctatcatcaactgtatcacgtactttcatggtgaggtgcc  960
                                   250
          Q  I  I  L  Y  L  G  I  L  L  S  H  A  N  S  M  M  N  P  I
961   acagatcatactgtatttgggcattctgctctcccatgctaactctatgatgaaccctat 1020
                                   270
          V  Y  A  Y  K  I  K  K  F  K  E  T  Y  L  L  I  F  K  T  Y
1021  tgtctatgcttataaaataaagaagttcaaggaaacctatcttttgatcttcaagaccta 1080
                                   290
          M  I  C  Q  S  S  D  S  L  D  S  S  T  E  *
1081  tatgatctgccagtcctctgattctttggactcaagcactgagtagacttctgagtagtt 1140
                                   310
1141  gtccttgaaagatgattcttcacccccattaacctccagattcaacatcagtaaacactt 1200
1201  gaggacctatttgcctgagccaagggctttcacatccttaatttcttctactgaggtggg 1260
1261  gagcatttgactggttgcctccaattgtatctcccctaacacccttcctgtaatccaatt 1320
1321  attttcttgtccttcttctctgattcactgttctggatgtctgacttgaggaaaatgtcc 1380
1381  tgctgttactactgtttgtgttcctccttcccaagcaagaggagaagttatggaatccga 1440
1441  aggaggccttgttgactcagagatgaaaagtcccagtcctgcgggacatgagtgtggtgg 1500
1501  cgactctgttccactccattgcagaatcacaccaagaaacctaccccagcagatgtcagg 1560
1561  gagatggtaggaacagtgtcacaaagggagacttaaactgctg                   1603
```

Fig. 1. Nucleotide and amino acid sequences of $cA_3$13.1, the canine A_3 adenosine receptor. The sequence has been deposited in GenBank (accession no. U54792).

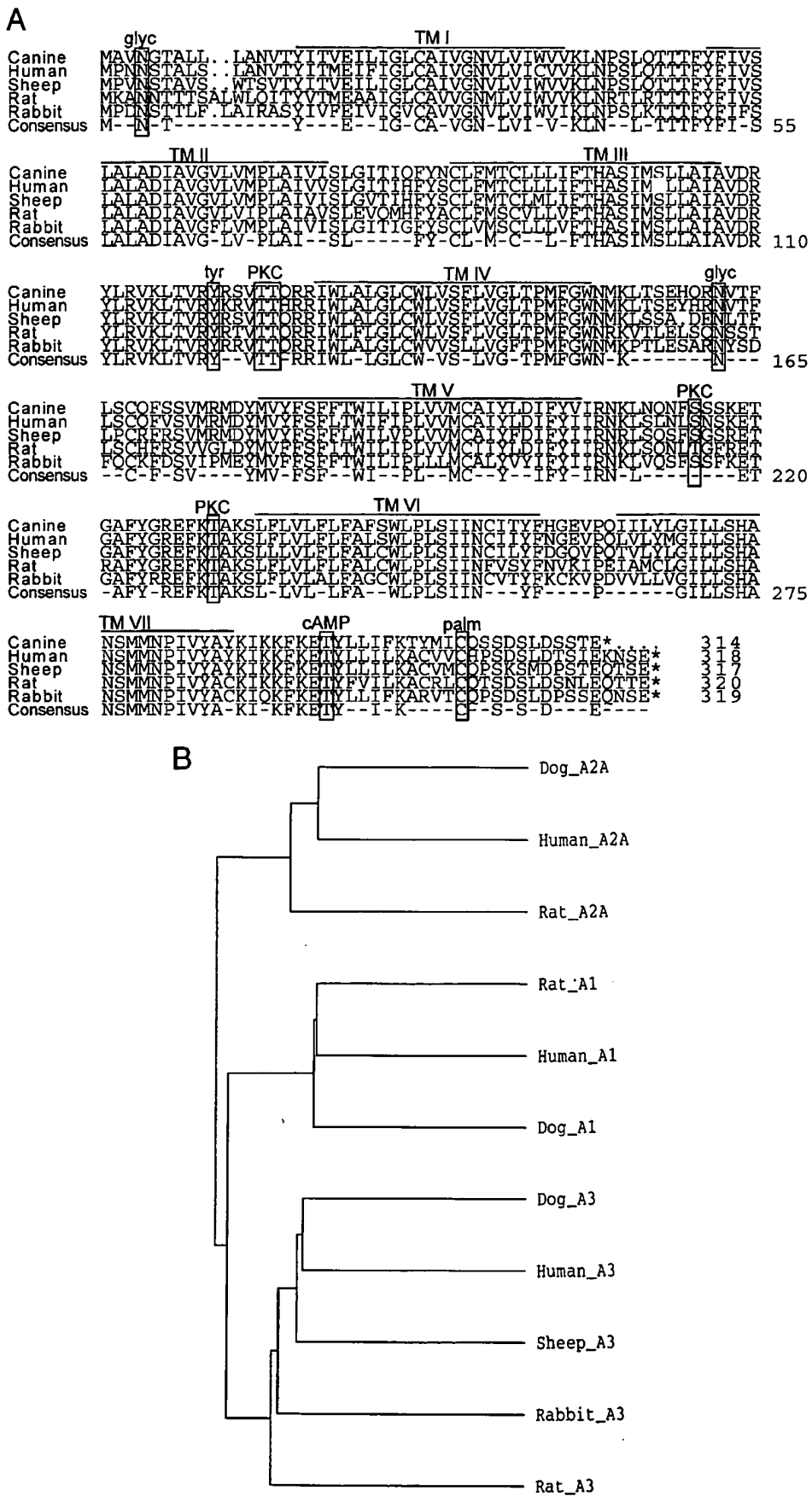


Fig. 2. Deduced amino acid sequence of $cA_3$13.1, the canine A_3 adenosine receptor. A, Alignment with human, sheep, rat, and rabbit A_3 adenosine receptor sequences. *Solid lines*, putative transmembrane (*TM*) domains with numbered designations (I–VII). *Dashes*, sequence gaps. Sites conserved among all species for possible *N*-linked glycosylation (*glyc*); phosphorylation by protein kinase C (*PKC*), cAMP-dependent protein kinase (*cAMP*), tyrosine kinases (*tyr*), and palmitoylation (*palm*) are designated. B, Dendogram illustrating species and subtype sequence differences among adenosine receptors. Distance along the horizontal axis is proportional to divergence of the amino acid sequences.

tween species, the greatest degree of homology lies within the transmembrane domains, and the least lies within the carboxyl tail. Of the five species of A_3 adenosine receptors cloned to date, the human receptor amino acid sequence is most similar to the canine and least similar to the rat. We noted previously that the human and rat A_3 adenosine receptors are unusually divergent for species homologs (12). In contrast, canine and human A_3 adenosine receptors show a degree of amino acid sequence homology that is similar to species differences among the other adenosine receptor subtypes (Fig. 2B).

Tissue distribution of A_3 mRNA. Northern blots probing for $cA_3$13.1 transcripts in several different canine tissues revealed two hybridizing bands of 1.9 and 2.7 kb (Fig. 3). Transcripts were most abundantly expressed in spleen, but high levels also were detected in lung and liver. Two major hybridizing bands were also observed in testes, but the sizes were 1.3 and 2.4 kb. Transcripts were not detected in heart or kidney by Northern analysis. Using the sensitive technique of RT-PCR, trace transcripts were observed in all six tissues studied (data not show). Transcripts for A_1, A_3, and A_{2B} adenosine receptors were detected by Northern blotting of BR cell poly(A)$^+$ mRNA (data not shown). The size of the A_3 transcripts, 1.9 and 2.7 kb, is the same as in dog spleen, lung, and liver. The A_{2B} transcript sizes are 1.6 and 1.8 kb and correspond to transcript sizes noted previously for A_{2B} receptor transcripts in mouse bone marrow-derived mast cells (21).

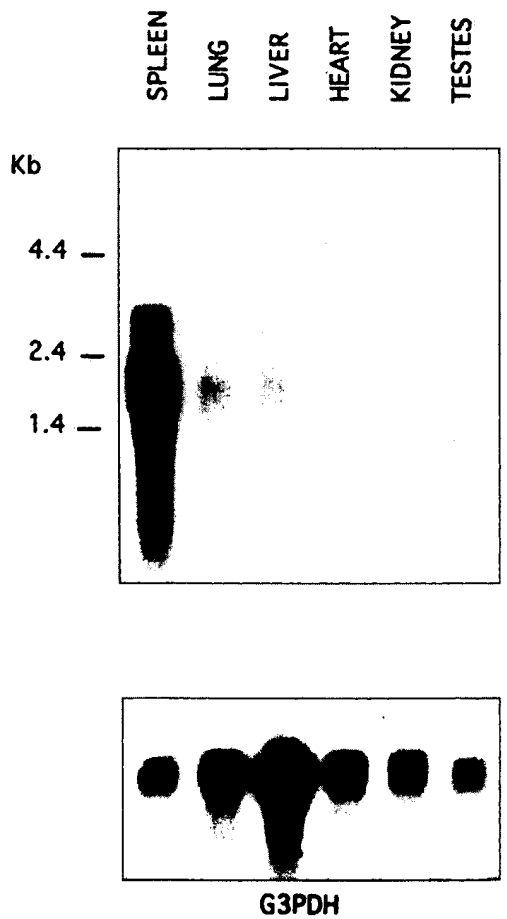


Fig. 3. Tissue localization of the canine A_3 adenosine receptor transcript. Northern blots of poly(A)$^+$ RNA (5 μg/lane) from six different dog tissues using a probe corresponding to the carboxyl-terminal tail and 3′ untranslated region of $cA_3$13.1. The same blot was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase transcript (*G3PDH*). RNA size markers are indicated.

Pharmacological characterization of canine A_3 adenosine receptors. Binding of [^{125}I]ABA was measured to membranes prepared from COS-7 cells transfected with $cA_3$13.1 (Fig. 4). Specific binding was absent in untransfected cells and was abolished by 1 μM nonradioactive I-ABA. *N*-Ethylmaleimide has been reported to alkylate G proteins in the $G_{i/o}$ family and cause them to become uncoupled from receptors. GTPγS and *N*-ethylmaleimide both reduced specific binding of [^{125}I]ABA to canine A_3 adenosine receptors by ~60%, indicating that the radioligand is an agonist and that G_i/G_o proteins couple to the A_3 receptor (Fig. 4A). In equilibrium binding studies, [^{125}I]ABA specific binding was consistently found to fit significantly ($p < 0.01$) better to a two-site than to a one-site model (22). The respective high and low affinity K_d values of [^{125}I]ABA are 0.53 ± 0.13 and 16.4 ± 0.8 nM, and B_{max} values are 250 ± 9 and 768 ± 123 fmol/mg of membrane protein. In the presence of 50 μM GTPγS, [^{125}I]ABA binds only to the low affinity site, with a K_d value of 17.4 ± 0.1 nM and a B_{max} value of 768 ± 123.0 fmol/mg of total protein. The conversion of receptors from two affinity states to a single low affinity state on the addition of GTPγS is most clearly illustrated by Scatchard analysis (Fig. 4C). These results suggest that the high affinity site reflects binding to G protein-coupled receptors and the low affinity site reflects binding to uncoupled receptors. A similar analysis indicates that [^{125}I]ABA also binds to two affinity states of recombinant rabbit A_3 adenosine receptors with K_D values of 1.2 and 34 nM (not shown). In contrast, in filtration assays, [^{125}I]ABA detects only the high affinity state of canine A_1 receptors transiently expressed in COS-7 cells (K_d = 2.67 ± 0.50 nM, B_{max} = 1275 ± 52 fmol/mg protein; Fig. 5), and specific binding is almost completely abolished by the addition of GTPγS (data not shown).

We next compared the binding properties of recombinant canine A_1 and A_3 adenosine receptors. Fig. 6 shows the results of competition binding studies with compounds found to be the most A_3 selective, IB-MECA and I-ABOPX, and those most A_1 selective, CPA and WRC-0571. K_i values of these and other compounds are summarized in Tables 1 and 2. These tables also summarize the relative affinities of competing compounds for canine A_1 and A_3 receptors. For the A_3 receptor subtype, two dissociation constants for agonists and single dissociation constants for antagonists were calculated as described in Experimental Procedures. The potency order of agonists for canine A_3 receptors was IB-MECA ≥ [^{125}I]ABA > PIA > APNEA > CPA > NECA > CGS 21680. IB-MECA is 58-fold selective for the A_3 over the A_1 adenosine receptor (high affinity sites), and WRC-0571 and CPA are 36- and 12-fold selective, respectively, for the A_1 over the A_3 receptor. The canine A_3 adenosine receptor binds antagonists with the potency order of I-ABOPX > CPX > XAC > BWA 1433 > WRC 0571 > 8-SPT (Table 2). Theophylline and enprofylline bind very weakly to canine A_3 adenosine receptors; a 100 μM concentration of these compounds reduces specific binding by only ~40%. I-ABOPX is 16-fold selective for canine A_3 over A_1 adenosine receptors.

Table 3 shows a comparison of the binding affinities of four

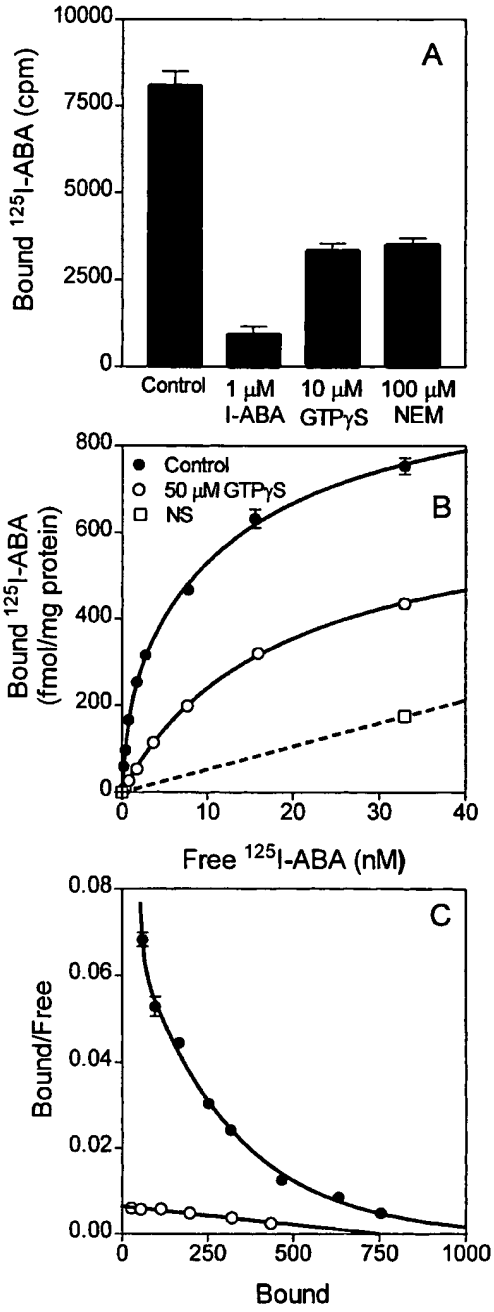


Fig. 4. Radioligand binding to recombinant canine A_3 adenosine receptors. A, Inhibition of [^{125}I]ABA (1.4 nM) binding to COS-7 cell membranes expressing $cA_3$13.1 by unradiolabeled I-ABA, GTPγS, and *N*-ethylmaleimide. B, Equilibrium specific and nonspecific (*NS*) binding of [^{125}I]ABA to transfected COS-7 cell membranes in the presence and absence of GTPγS. C, Scatchard transformation of the specific binding data shown in B. For B and C, control data were fit optimally to two-site equations as described in Experimental Procedures. Values are mean ± standard error of triplicate determinations (25 μg of membrane protein/tube); where omitted, standard error bars are smaller than the symbols. The results are typical of three experiments. Binding parameters are summarized in Table 1.

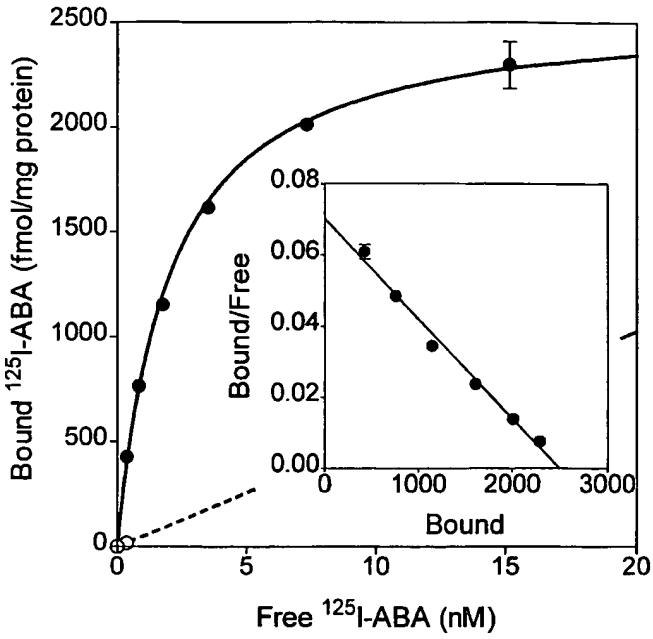


Fig. 5. Radioligand binding to recombinant canine A_1 adenosine receptors (RDC7). Equilibrium specific (●) and nonspecific binding (○) binding of [^{125}I]ABA to transfected COS-7 cell membranes is plotted. *Inset*, Scatchard transformation of the specific binding. Values are mean ± standard error of triplicate determinations (10 μg of membrane protein/tube); where omitted, standard error bars are smaller than the symbols. The results are typical of three experiments. Binding parameters are summarized in Table 1.

xanthines with those of recombinant human, canine, rabbit, and rat A_3 receptors and confirms that there are marked species differences in the binding affinities of xanthine antagonists. Of the four xanthines examined, the average ratio of binding affinities is 9.6 (canine/human), 5.0 (rabbit/human), and 212 (rat/human). The canine A_3 receptor binds CPX with higher affinity than any of the other species examined.

[^{125}I]ABA binding to canine BR mastocytoma cell membranes. Because both A_1 and A_3 adenosine receptor transcripts were detected in canine BR cells, we next determined whether [^{125}I]ABA binding to A_1 and/or A_3 receptors could be detected in membranes prepared from these cells. Little specific binding could be detected to crude membranes, but the binding of 0.4 nM radioligand to an enriched P2 membrane preparation was 80% specific (Fig. 7). Of the [^{125}I]ABA binding site on BR cell membranes, 24 ± 3% (three experiments) bind WRC-0571 with low affinity (IC_{50} = 22 ± 9 μM) characteristic of A_3 receptors; the remainder bind WRC-0571 with high affinity (IC_{50} = 114 ± 38 nM) characteristic of A_1 receptors (Fig. 7, Table 2). When added at 0.4 nM, [^{125}I]ABA labeled only 2.4 fmol/mg of protein of A_3 receptors in the P2 membranes of BR cells, suggesting the density of A_3 receptors on BR cells is low.

Characterization of the adenosine receptor that causes degranulation of canine mastocytoma cells. Adenosine agonists have been shown to enhance A23187 (calcimycin)-stimulated degranulation of several types of mast cells, including murine bone marrow-derived mast cells, human lung mast cells, RBL-2H3 cells, and rat peritoneal mast cells (23, 24). Fig. 8 shows the effect of increasing concentrations of Ca^{2+} ionophore to elicit β-hexosaminidase release from BR cells when administered alone or in combination with the nonselective adenosine receptor agonist NECA (10 μM). When administered alone, A23187 evokes a maximal release of ~15.6% of the total cellular content after

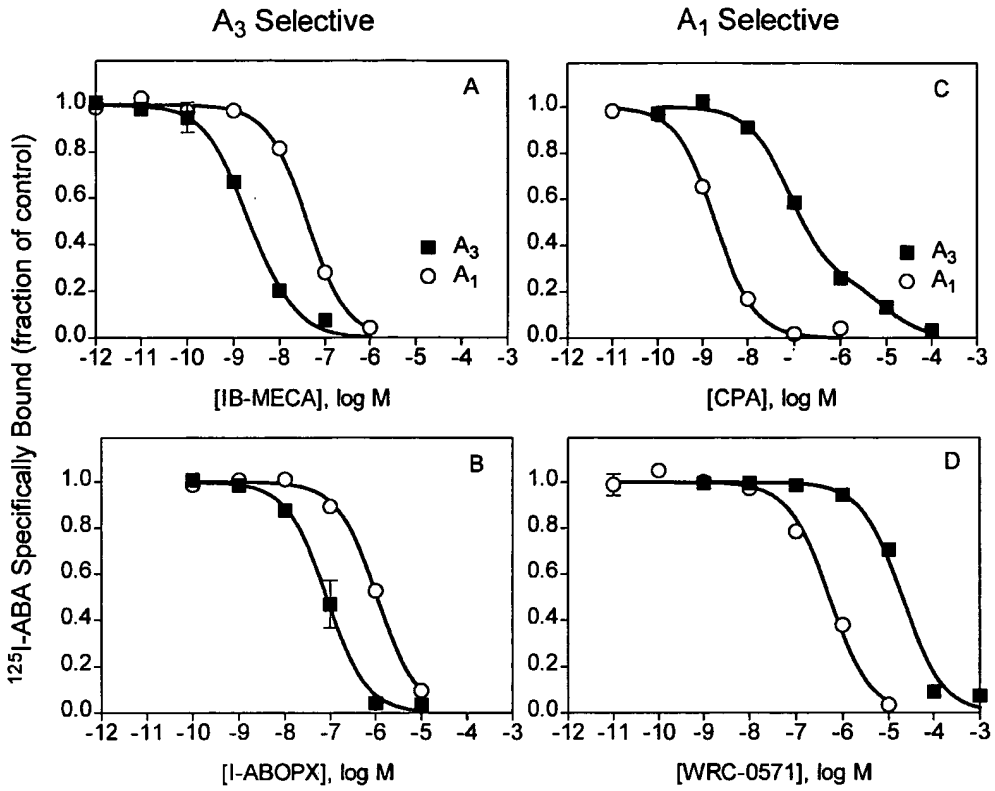


Fig. 6. Competition for [^{125}I]ABA binding to COS-7 cell membranes derived from cells transfected with canine A_3 or A_1 adenosine receptors. Binding is plotted as the fraction of control specific binding (>90% of total binding). Values are mean ± standard error of triplicate determinations from three experiments. Protein, 25 μg/tube (A_3) or 10 μg/tube (A_1), [^{125}I]ABA, 150,000–300,000 cpm/tube (0.4–0.8 nM). The binding of [^{125}I]ABA and competing ligands was fit to one or two binding sites as described in Experimental Procedures.

20 min of stimulation. NECA (10 μM) alone also stimulates β-hexosaminidase release (5.81 ± 0.59%), and costimulation with NECA and A23187 increases β-hexosaminidase release to 28.2 ± 1.8%. NECA decreases the EC_{50} for A23187 from 0.32 ± 0.06 to 0.13 ± 0.08 μM.

We next conducted experiments to determine whether NECA acts at receptor sites on the cell surface or at intracellular sites. To test the possibility that NECA may gain access into BR cells via facilitated uptake and influence the degranulation response via an intracellular mechanism, BR cells were preincubated for 15 min with NBTI (1 μM), an inhibitor of nucleoside transport. As shown in Fig. 8B, NBTI did not influence the concentration response of NECA to stimulate β-hexosaminidase release. As additional evidence that NECA acts at cell surface adenosine receptors, we added XAC, a nonselective antagonist of all subtypes of canine adenosine receptors, including A_3 (see Table 2). XAC completely abolished the stimulatory effect of NECA (Fig. 8B). Additional experiments were performed with NBTI and XAC during costimulation with a combination of A23187 and NECA. As in the absence of A23187, NBTI had no effect, and XAC abolished NECA-mediated degranulation responses (data not shown).

Pertussis toxin blocks the inhibition of cAMP accumulation in CHO-K1 cells that is mediated by recombinant rat A_3 adenosine receptors (2). Pertussis intoxication of rats also reduces a putative A_3 adenosine receptor-mediated hypotensive response (25). These data suggest that A_3 adenosine receptors functionally couple to G_i/G_o proteins; therefore, we examined the effect of pretreating BR cells with pertussis toxin on the ability of NECA to stimulate degranulation (Fig. 9). For these experiments, BR cells were cultured in serum-free medium with 0.3 or 1 μg/ml of pertussis toxin for 24 hr.[1] We found that cells cultured in serum-free medium released a greater amount of β-hexosaminidase in response to 1 μM A23187 (~25–35% without serum versus ~15% with serum). Pretreatment of cells with either concentration of pertussis toxin did not prevent NECA-stimulated degranulation. These data suggest that neither A_1 nor A_3 adenosine receptors are solely responsible for adenosine-mediated degranulation of BR cells.

We next determined the potency order of various adenosine analogs to stimulate β-hexosaminidase release from BR cells. In addition to NECA, we examined (*R*)-PIA (A_1 selective), CGS 21680 (A_{2A} selective), and IB-MECA (A_3 selective). Experiments were performed in the presence or absence of 1 μM A23187 (results are illustrated in Fig. 10 and summarized in Table 4). The potency order of agonists to stimulate canine mast cell degranulation, NECA > PIA > CGS-21680 > IB-MECA, differs from the potency order of these compounds for binding to canine A_3 adenosine receptors, IB-MECA > PIA > NECA > CGS-21680. IB-MECA has very little stimulatory effect on BR cell degranulation and, when added at 10 μM, IB-MECA inhibits degranulation.

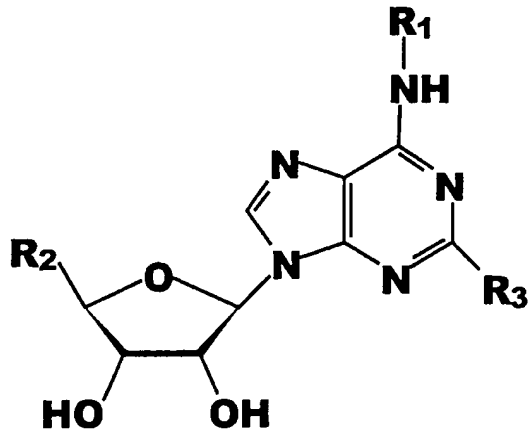
The observed potency order for adenosine analogs to stimulate BR mast degranulation is similar to the potency order reported by Brackett and Daly (26) for activating A_{2B} receptors on NIH 3T3 fibroblasts and the potency order for binding to recombinant human A_{2B} receptors (27). Furthermore, A_{2B} receptors in general have low affinity for agonists, similar to their low affinity for stimulating degranulation of BR cells

[1] Serum-free medium was used to avoid the potential for neutralization of pertussis toxin by anti-toxin antibodies that exist within some lots of serum.

TABLE 1
Dissociation constants of agonists for the canine A_3 (cA_3 13.1) and A_1 adenosine receptors determined in binding assays

R_1	R_2	R_3	A_3 receptor (cA_3 13.1)		A_1 receptor	A_1/A_3[b]	A_3/A_1[b]
			K_{i1}	K_{i2}	K_i		
					nM		
IB-MECA	3-Iodobenzyl	Methylcarboxamido	H	0.53 ± 0.13	89.5 ± 41.3	30.6 ± 2.10	57.7
I-ABA	3-Iodo-4-aminobenzyl	$HO-CH_2$	H	0.71 ± 0.09[a]	16.4 ± 0.80[a]	2.67 ± 0.50	3.76
APNEA	4-Aminophenylethyl	$HO-CH_2$	H	8.10 ± 0.13	250 ± 72	13.9 ± 2.12	1.72
(*R*)PIA	Phenylisopropyl	$HO-CH_2$	H	2.71 ± 0.47	472 ± 36	2.31 ± 0.10	1.17
NECA	H	Ethylcarboxamido	H	34.3 ± 4.9	10,300 ± 4,060	2.92 ± 0.30	11.8
CPA	Cyclopentyl	$HO-CH_2$	H	21.9 ± 4.2	3,840 ± 1,240	1.82 ± 0.39	12.0
CGS 21680	H	Ethylcarboxamido	(4-Carboxyethyl)-phenyethylamino	398 ± 16	70,600 ± 710		

K_i values (nM ± standard error of three to six experiments) obtained from competition experiments using [^{125}I]ABA as the radioligand; K_{i1} and K_{i2}, dissociation constant for the high and low affinity sites for the A_3 receptor, respectively; [a] K_D values for [^{125}I]ABA determined in equilibrium binding assays; [b] ratio of the K_{i1} values for the A_3 receptor and K_i value for the A_1 receptor. The procedures for deriving K_{i1} and K_{i2} are described in the text.



(Table 4). Because A_{2B} receptor transcript is found in BR cells, we postulated that A_{2B} adenosine receptors are expressed on BR cells and, when activated, stimulate degranulation. To test this hypothesis, we investigated the effects of enprofylline, which we recently identified as a selective antagonist of recombinant human A_{2B} receptors (27). Concentration-response curves for degranulation in response to NECA were generated in the presence of 10, 50, and 250 μM enprofylline. As shown in Fig. 11A, increasing concentrations of enprofylline produced parallel rightward shifts of the concentration-response curves for β-hexosaminidase release. Schild regression analysis revealed a slope close to unity (1.12 ± 0.45), suggesting that enprofylline acts as a competitive antagonist at a single receptor subtype. The K_D value of enprofylline was estimated to be 7.8 ± 3.3 μM. This value is almost identical to the K_I value of enprofylline for binding to human A_{2B} receptors and is well below the K_I value of enprofylline for canine A_1 or A_3 receptors. Interestingly, the K_i value of enprofylline for A_{2B} receptors lies within the therapeutic range of this compound as an antiasthmatic therapeutic agent (28). Because enprofylline also inhibits cAMP phosphodiesterase, we evaluated the effects of another phosphodiesterase inhibitor, Ro 20–1724, on NECA-induced β-hexosaminidase release. Ro 20–1724 is a nonxanthine that does not bind to adenosine receptors. As shown in Fig. 11B, Ro 20–1724 has no effect on NECA-induced degranulation. The data are consistent with the possibility that enprofylline blocks BR cell degranulation by binding to A_{2B} adenosine receptors.

Second messenger responses evoked by A_{2B} receptor activation in canine mastocytoma cells. We next measured second messenger responses to adenosine receptor activation in BR cells. Unlike A_3 adenosine receptors, which are inhibitory to adenylyl cyclase, A_{2B} adenosine receptors stimulate adenylyl cyclase (26). We measured changes in intracellular levels of cAMP, Ca^{2+}, and $InsP_3$ in response to NECA, CGS 21680, and IB-MECA. As shown in Fig. 12, NECA, but not CGS 21680 or IB-MECA, produces a concentration-dependent increase in intracellular levels of cAMP. The absence of cAMP accumulation in response to CGS-21680 indicates that accumulation of the cyclic nucleotide in response to NECA is not mediated by A_{2A} adenosine receptors. The calculated EC_{50} for NECA to increase cAMP in BR cells is 0.9 ± 0.2 μM, which is similar to the EC_{50} value for NECA to stimulate β-hexosaminidase release (0.6 ± 0.3 μM), suggesting that both responses are mediated by the same receptor subtype. NECA (1 μM) also increased intracellular levels of Ca^{2+} and $InsP_3$, whereas 1 μM CGS -21680 or 1 μM IB-MECA had little effect (Fig. 12). Small responses to CGS 21680 and IB-MECA to influence cAMP, Ca^{2+}, or $InsP_3$ suggest that selective activation of A_{2A} or A_3 receptors has little effect on cAMP or Ca^{2+} signaling in canine BR mastocytoma cells.

Enprofylline competitively antagonized the increases in intracellular levels of cAMP and Ca^{2+} produced by NECA (Fig. 13). By Schild regression analysis (slope = 0.81 ± 0.15), the K_D value of enprofylline was estimated to be 4.7 ± 3.2 μM from the cAMP response and 15 μM from the Ca^{2+} response,

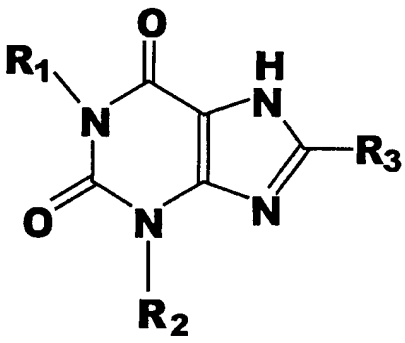
TABLE 2

Dissociation constants of antagonists for the canine A_3 (cA_3 13.1) and canine A_1 adenosine receptors determined in binding assays

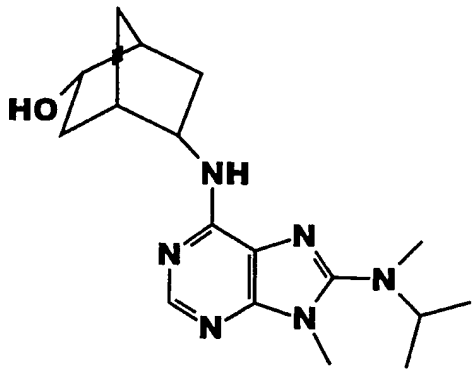
All structures except WRC-0571 are xanthines.

	Xanthine			K_i		A_1/A_3	A_3/A_1
	R_1	R_2	R_3	A_3	A_1		
				nM			
I-ABOPX	Propyl	3-Iodo-4-aminobenzyl	Phenyl-oxyacetate	37.5 ± 11	601 ± 99	16.0	
XAC	Propyl	Propyl	Phenyl-(2-aminoethyl)aminocarbonylmethyloxy	138 ± 22	159 ± 12	1.2	
8-SPT	Methyl	Methyl	Phenyl-sulfol	25,300 ± 4,200	6,460 ± 590		3.9
CPX	Propyl	Propyl	Cyclopentyl	115 ± 10	11.4 ± 2.1		10.1
BW-A1433	Propyl	Propyl	Phenyl-acrylate	1,880 ± 190	132 ± 11		14.2
WRC-0571	(Nonxanthine)			17,200 ± 2,100	484 ± 41		35.4
Theophylline	Methyl	Methyl	H	>100,000			
Enprofylline	H	Propyl	H	>100,000	>100,000		

K_i values obtained from competition experiments using [^{125}I]ABA as the radioligand; all values are reported as mean ± standard error of three to six experiments.



Xanthines



WRC-0571

TABLE 3

Species differences in the binding of xanthines to A_3 adenosine receptors

	K_i				K_i ratio (other species/human)		
	Human	Canine	Rabbit	Rat	Canine	Rabbit	Rat
	nM						
I-ABOPX	18	37.5	179	1,500	2.1	9.9	83
BW-A1433	55	1,880	384	15,000	34	7.0	272
XAC	71	138	106	29,000	1.9	1.5	408
CPX	509	115	708	43,000	0.23	1.4	84

K_i values were obtained from competition experiments using recombinant receptors from the indicated species with [^{125}I]ABA as the radioligand for three to six experiments. In all cases, the standard error was <30% of the mean.

values similar to the K_D value estimated for enprofylline to inhibit NECA-stimulated β-hexosaminidase release (7.8 μM). These results suggest that A_{2B} receptors in BR cells are positively coupled to adenylyl cyclase and phospholipase C.

Discussion

We cloned and characterized a canine A_3 adenosine receptor cDNA designated $cA_3$13.1 from canine BR mastocytoma cells. The clone is homologous to A_3 receptors cloned from other species. Transcripts for A_1 and A_{2B} adenosine receptors also were detected in BR cells, and evidence of A_1, A_{2B}, and A_3 receptor expression was found on the basis of radioligand binding or functional assays. The A_{2B} receptor predominates in the regulation of BR cell degranulation, as discussed in detail below.

The tissue distribution of A_3 adenosine receptor transcript in dog is similar to the human distribution (3), with highest levels expressed in spleen, followed by lung and liver. The tissue distribution is different from rat, in which transcript is much more abundant in testes than in other tissues (1). In terms of sequence homology and pharmacology, the canine A_3 adenosine receptor is more similar to the human than to the rat A_3 receptor.

The results of radioligand binding assays with the A_1/A_3 agonist [^{125}I]ABA indicate that the canine A_3 adenosine receptor binds to both G protein-coupled and -uncoupled receptors with K_D values that differ by ~30-fold. The potency order of agonists for the A_3 receptor, IB-MECA > R-PIA ≥ NECA > CPA, is consistent among species. In all species, IB-MECA and CPA are A_3 and A_1 selective, respectively. Specific binding of [^{125}I]ABA to the uncoupled conformation of the canine A_3 aden-

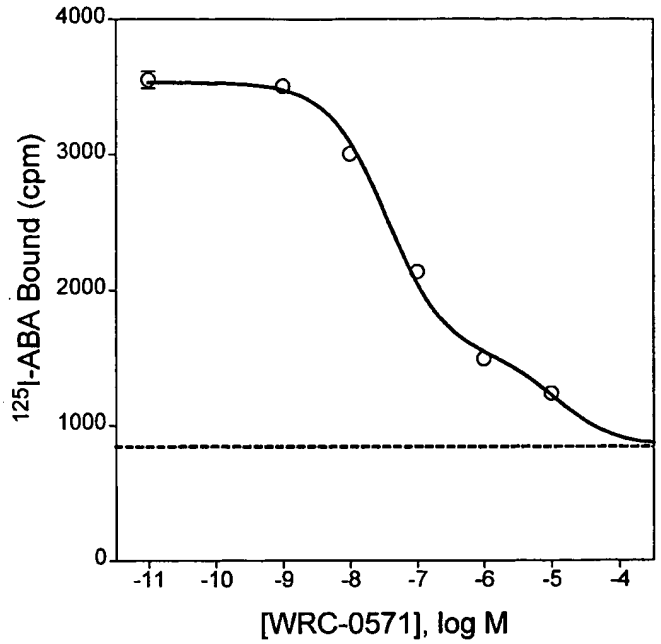


Fig. 7. Competition by WRC-0571 for [^{125}I]ABA binding to enriched plasma membranes derived from BR cells. P2 membranes were prepared as described in Radioligand Binding Studies. Binding was determined in triplicate to membranes containing 156,000 cpm (0.4 nM) [^{125}I]ABA, 60 μg of P2 membrane protein, and various concentrations of WRC-0571. Nonspecific binding (10 μM I-ABA) is 846 ± 18 cpm (*dashed line*). Standard error bars are smaller than the symbols. The data were fit to a two-site model probably reflecting WRC-0571 binding to A_1 and A_3 adenosine receptors. Binding parameters from triplicate experiments are summarized in the text.

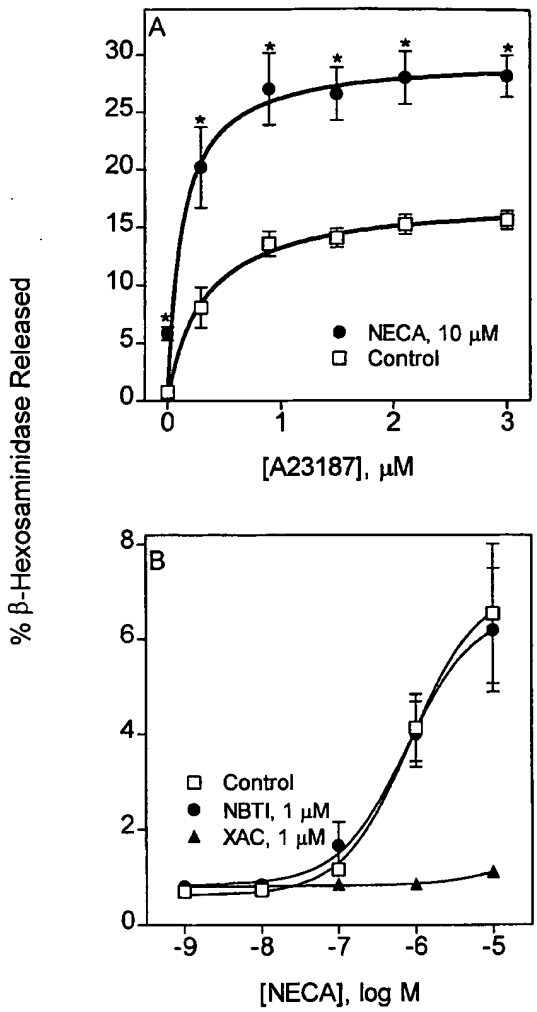


Fig. 8. Stimulation of β-hexosaminidase release from BR cells by A23187 and NECA. A, Cells (300,000/tube) were stimulated with increasing concentrations of A23187 in the presence or absence of 10 μM NECA for 20 min. β-Hexosaminidase released into the supernatant was measured as described in Experimental Procedures. B, Effects on NECA-stimulated β-hexosaminidase release of pretreatment of cells for 15 min with NBTI (1 μM) or XAC (1 μM). Data are pooled from three separate experiments, each assayed in triplicate. *, Different from control ($p < 0.05$).

osine receptor distinguishes the canine A_3 receptor from the uncoupled canine A_1 receptor, which has too low affinity for [^{125}I]ABA binding to be detected in filtration assays. However, [^{125}I]ABA binds with 10 times higher affinity to bovine than to canine A_1 receptors, and the radioligand can detect two affinity states of the bovine A_1 adenosine receptor [K_d = 0.09 and 10.4 nM (29)]. The detection in filtration assays of two affinity states of A_3 receptors complicates the analysis of competition binding assays because the radioligand binds with two affinities and competing agonists and antagonists bind with two or one affinities, respectively. To calculate the K_i values of competing compounds required the derivation of nonstandard analytical procedures (see Analysis of Binding Data). As summarized in Table 1, I-ABA and IB-MECA both bind with high affinity ($K_D < 1$ nM) to the G protein-coupled conformation of canine A_3 receptors. Failure to analytically resolve the two agonist affinity states in radioligand binding assays will result in underestimation of high affinity agonist dissociation constants as well as errors in the calculation of the dissociation constants of competing compounds based on the Cheng and Prusoff formula (30). It is notable that in the range of 0.1–1 μM, compounds that often are used as selective agonists of A_1 receptors (CPA) or A_{2A} receptors (CGS-21680) also will bind to canine A_3 receptors. Hence, caution must be taken in attributing functional responses of these compounds to particular adenosine receptor subtypes.

This study confirms and extends the observation that there are substantial species differences in the binding of xanthines to A_3 adenosine receptors. The rat A_3 adenosine receptor, the first A_3 adenosine receptor to be cloned, was originally reported not to bind xanthine antagonists (2). Subsequent studies have shown that xanthines bind weakly to the rat receptor. The most potent xanthine antagonist, I-ABOPX, binds to the rat A_3 receptor with a K_I value of 1.5 μM. In contrast, sheep, human, and canine A_3 receptors bind I-ABOPX with 80–500 times higher affinity (3, 4).

CPX is widely regarded as a selective antagonist of A_1 adenosine receptors. Although CPX is >250-fold selective for human A_1 over A_3 receptors (27), this selectivity drops to only 10-fold in the case of canine receptors. This is partly due to the fact that compared with human and sheep A_3 receptors, canine A_3 receptors bind CPX with relatively high affinity. In addition, canine A_1 adenosine receptors have lower affinity than other species for CPX. Consequently, CPX is not particularly useful for discriminating between A_1 and A_3 receptor-mediated responses in the dog. A preferable compound for this purpose is WRC-0571, an A_1-selective nonxanthine antagonist that is >4000-fold selective for human A_1 over A_3 receptors (31). Although WRC-0571 binds with much lower

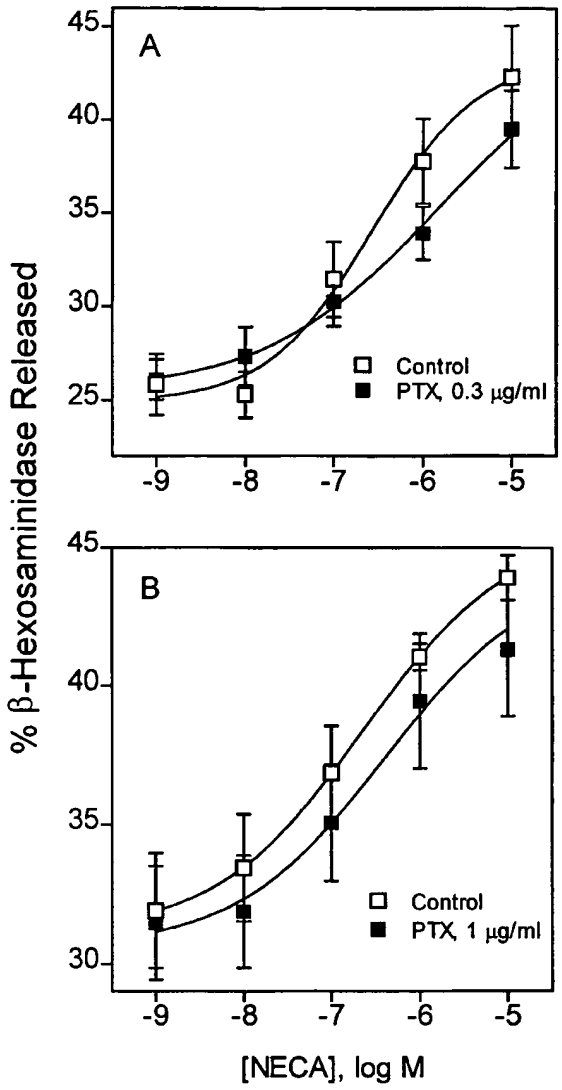


Fig. 9. Effect of pretreatment of BR cells with pertussis toxin (*PTX*) on NECA-stimulated degranulation. Cells were pretreated for 24 hr with 0.3 μg/ml (A) or 1 μg/ml (B) pertussis toxin and then stimulated for 20 min with 1 μM A23187 and various concentrations of NECA. Data are pooled from three separate experiments, each assayed in triplicate.

affinity to canine A_1 receptors (K_I = 484 nM) than to human A_1 receptors (K_I = 3 nM), it still is 35-fold selective as an antagonist of canine A_1 over A_3 receptors. Species differences in binding affinity also are significant for BW-A1433 [8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine], which is sometimes used as a A_3 receptor antagonist on the basis of its moderate affinity for sheep and human A_3 receptors (3, 4). BW-A1433 is a relatively weak and nonselective antagonist of canine A_3 receptors, binding with 10-fold lower affinity to canine than to human A_3 receptors.

Enprofylline, an antiasthmatic agent that has moderate affinity for human A_3 receptors [K_I = 156 ± 110 μM (27)], binds poorly to the canine A_1 and A_3 receptors (K_I > 100 μM). Because enprofylline binds to the human A_{2B} adenosine receptors with a K_I value of 7 μM (27), the compound was evaluated in this study to discriminate between canine A_{2B} and A_1 or A_3 adenosine receptor-mediated responses. Inasmuch as the canine A_3 receptor clone was isolated from a

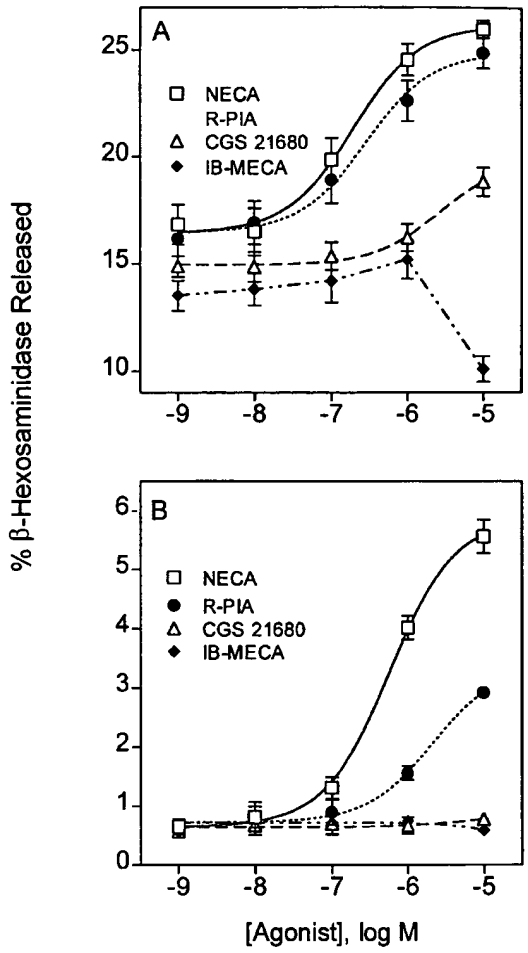


Fig. 10. β-Hexosaminidase release from BR cells in response to adenosine receptor agonists. A, Cells were stimulated with adenosine agonists and 1 μM A23187 for 20 min. B, Cells were stimulated with adenosine agonists alone. Values are mean ± standard error of data pooled from three separate experiments, each assayed in triplicate.

TABLE 4

Potency of adenosine analogs to stimulate β-hexosaminidase release from canine BR mastocytoma cells mean ± SEM, n = 3

	EC_{50}[a]	
	Without A23187	With 1 μM A23187
	μM	
NECA	0.58 ± 0.27	0.19 ± 0.03
(*R*)PIA	11.5 ± 2.5	0.47 ± 0.14
CGS 21680	[b]	32.4 ± 33.5
IB-MECA	[b]	[b]

[a] Data were pooled from three independent experiments.
[b] No β-hexosaminidase release was observed.
Values are mean ± standard error for three determinations.

canine mastocytoma cDNA library, we anticipated that the A_3 receptor subtype would be responsible for stimulating the release of granule-associated mediators. However, A_{2B} and A_1 as well as A_3 transcript were found in BR cells, and low levels of A_1 and A_3 receptor binding sites could be detected on enriched plasma membranes prepared from the canine mastocytoma cells. Nevertheless, several lines of evidence indicate that BR cell degranulation requires activation of the A_{2B} but not the A_1 or A_3 adenosine receptor: (i) degranulation of BR cells is not prevented by pretreatment of cells with per-

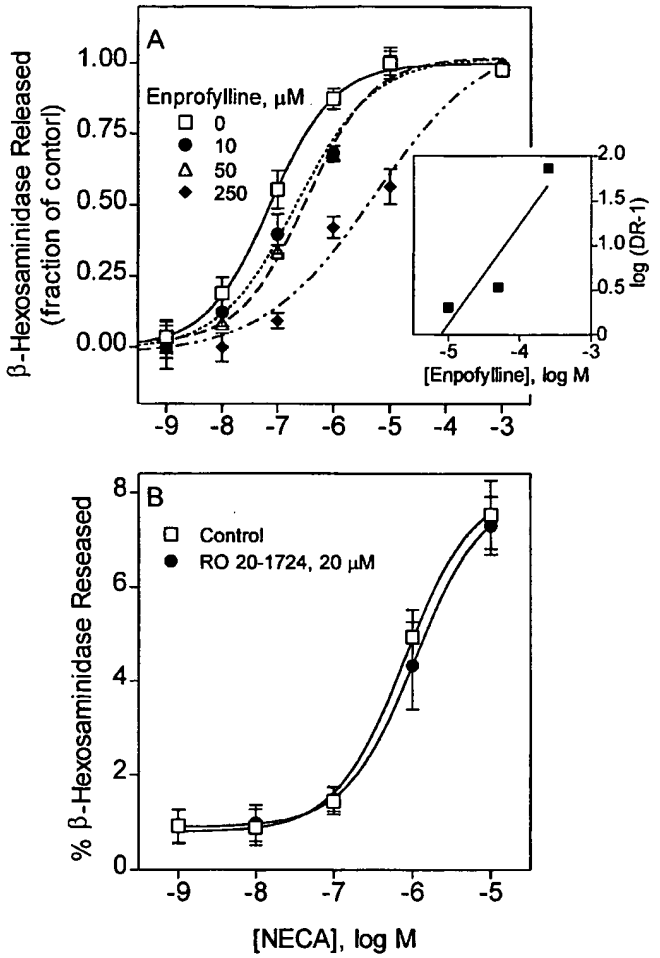


Fig. 11. Effects of enprofylline and Ro 20–1724 on β-hexosaminidase release from BR cells. A, Antagonistic effect of enprofylline on NECA-induced β-hexosaminidase release from BR cells costimulated with 1 μM A23187 and various concentrations of NECA. *Inset*, Schild plot; for enprofylline, pA_2 = 5.1. B, Cells were pretreated with or without 20 μM Ro 20–1724 for 15 min and then stimulated with NECA. Data are mean ± standard error of three determinations; similar results were observed in a replicate experiment.

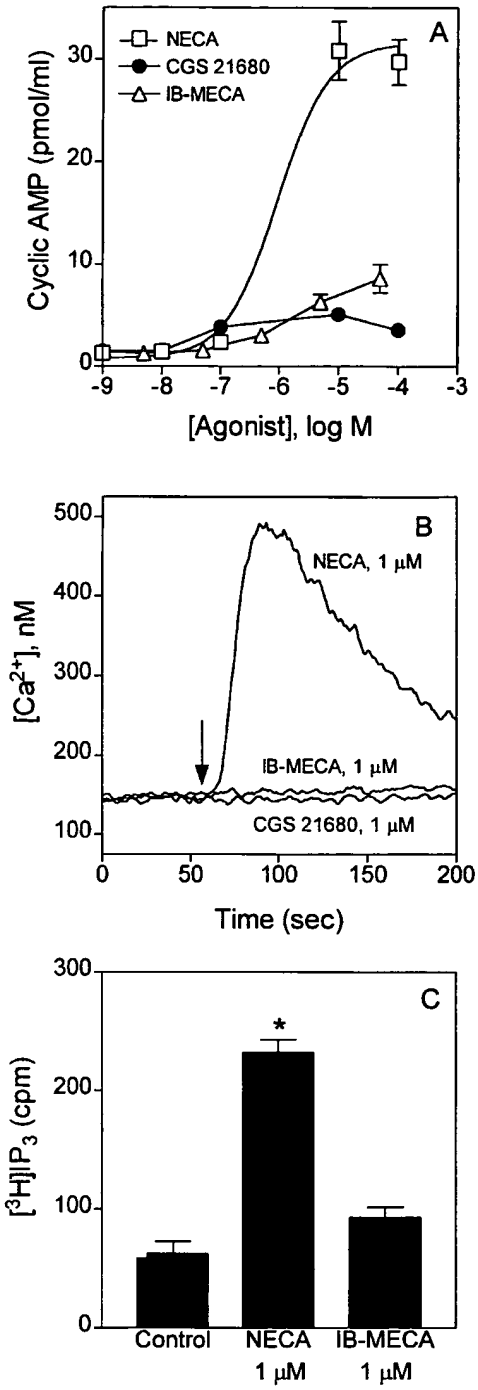


Fig. 12. Changes in intracellular levels of cAMP, Ca^{2+}, and $InsP_3$ in BR cells during stimulation with adenosine agonists. A, cAMP levels were measured in suspended BR cells incubated for 10 min with 20 μM Ro 20–1724. B, Ca^{2+} levels were measured in suspended cells loaded with FURA-2/AM. C, $InsP_3$ levels were measured in cells prelabeled with *myo*-[^{3}H]inositol. All incubations included 1 unit/ml adenosine deaminase. Data are mean ± standard error of three experiments. *, Greater than control and IB-MECA ($p < 0.01$).

tussis toxin; (ii) the response is blocked by enprofylline with a pA_2 value near 5, an affinity similar to that of human A_{2B} receptors and higher that the affinity of enprofylline for canine A_1 or A_3 receptors; (iii) the potency order of agonists to stimulate degranulation, NECA > PIA > CGS-21680 > IB-MECA, differs from the potency order of these compounds to bind to recombinant canine A_3 adenosine receptors; and (iv) NECA, but not CGS-21680, elevates cAMP, which is consistent with the existence of functional A_{2B} receptors on BR cells.

It was somewhat unexpected that A_{2B} adenosine receptors seem to couple to Ca^{2+} mobilization in canine mastocytoma cells inasmuch as A_{2B} adenosine receptors have been shown to couple to stimulation of cAMP accumulation (26). Apparent dual coupling to cAMP and Ca^{2+} has also been noted in HEK 293 cells stably transfected with recombinant human A_{2B} receptors,[2] and it is significant in this regard that the expression of recombinant rat A_{2B} receptors in *Xenopus laevis* oocytes results in the appearance of adenosine-mediated Ca^{2+}-dependent Cl^- current (32). Dual coupling of G protein coupled receptors to G_s and $G_{q/11}$ is not unprecedented. For example, the human prostacyclin receptor also displays such dual coupling (33). Coupling of A_{2B} adenosine receptors to a

[2] X. Jin and J. Linden, unpublished observations.

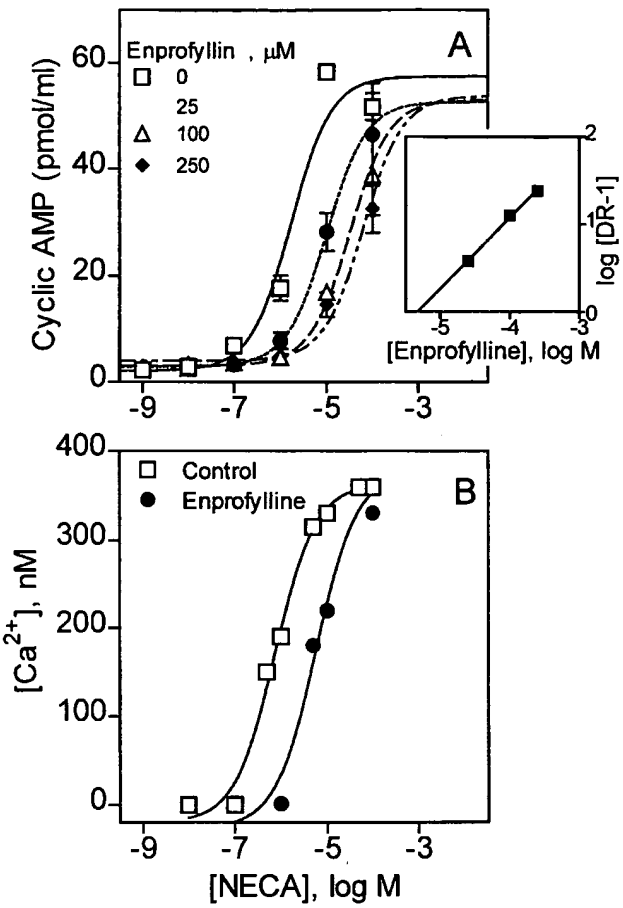


Fig. 13. Antagonistic effects of enprofylline on second messenger responses in canine BR cells. A, cAMP responses to NECA and enprofylline. *Inset*, Schild plot; for enprofylline, $pA_2 = 5.3$. B, Concentration-dependence of Ca^2 responses to NECA with and without enprofylline (100 μM); the EC_{50} values for NECA are 760 and 5725 nM in the absence and presence of enprofylline, respectively. The pA_2 value for enprofylline is estimated to be 4.8. Results are typical of three experiments.

Ca^{2+}-mobilizing G protein resistant to pertussis toxin ($G_{q/11}$) may be essential for triggering BR cell degranulation because agents that elevate cAMP in various kinds of mast cells, including agonists of A_{2A} adenosine receptors, either have no effect or are inhibitory to degranulation (34, 35).

The conclusions of previous studies have been inconsistent regarding the adenosine receptor subtype that mediates mast cell degranulation. Recent DNA antisense experiments suggest that activation of A_1 adenosine receptors may contribute to bronchoconstriction in a rabbit model of asthma (36). However, the low potency of various A_1-selective xanthines to block histamine release from asthmatic human lung fragments (10) and the low potency of enprofylline to block human A_1 adenosine receptors (27) are consistent with the participation of A_{2B} and/or A_3 receptors in human disease. The A_3 adenosine receptor has been implicated in the degranulation of RBL 2H3 rat mast cells and in triggering vascular responses[3] secondary to degranulation of mast cells in the hamster cheek pouch (9, 37) and the pithed rat (25). A_{2B} adenosine receptors seem to mediate the degranulation of murine bone marrow-derived mast cells (21), and although pretreatment of RBL 2H3 rat mast cells with pertussis toxin abolishes NECA-mediated degranulation, Ca^{2+} mobilization in these cells requires activation of G_{i3} or G_q (38). Moreover, activation of phosphoinositide breakdown in RBL 2H3 cells is not well correlated with the affinity of adenosine analogs for A_3 adenosine receptors (39). The treatment of murine bone marrow mast cells with pertussis toxin produces a decrease in the potency of adenosine to enhance degranulation in response to A23187, similar to the result in the current study with canine mastocytoma cells. Pretreatment of murine mast cells with pertussis toxin fails to reduce adenosine-mediated Ca^{2+} mobilization, which is consistent with an A_{2B}-mediated, but not an A_3-mediated, response (40). In the human HMC-1 mast cell leukemia line, the ability of 300 μM enprofylline to block NECA-stimulated interleukin-8 release was taken as evidence that this response also is mediated by A_{2B} adenosine receptors (41). The current study tends to substantiate previously published reports that suggest the receptor subtype primarily responsible for adenosine-mediated mast cell degranulation is variable. It is not yet clear whether this variability depends on species, tissue source, or environmental factors that affect signaling cascades and/or the phenotype of various mast cells. The results of this study indicate that A_{2B} receptors play a major role in the regulation of mast cell degranulation but are consistent with possible participation of multiple adenosine receptor subtypes and multiple G proteins.

The canine A_3 adenosine receptor described in this study is structurally and pharmacologically more similar to human A_3 receptors than are A_3 receptors from rodent species. This finding, along with the fact that it seems that murine bone marrow mast cells, canine mastocytoma, and human HMC-1 leukemic mast cells are regulated by adenosine, primarily via A_{2B} receptors, raises the possibility that canine models of asthma may be better predictors of human disease than rodent models. It will be important to determine which adenosine receptor subtype or subtypes are responsible for facilitating mast cell degranulation in the asthmatic human lung. Once the predominant receptor or receptors are identified, novel antagonists superior to theophylline and enprofylline for the treatment of asthma may be developed.

Acknowledgments

cAMP determinations were made at the University of Virginia Radioimmunoassay Core. We are grateful to Fereydoun Sajjadi (Gensia), Marlene Jacobson (Merck), and Scott Kennedy (Pfizer) for their gifts of A_3 adenosine receptor cDNAs and to J. H. Butterfield (Mayo Clinic, Cleveland, OH) for HMC-1 cells.

References

1. Meyerhof, W., R. Müller-Brechlin, and D. Richter. Molecular Cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* **284**:155–160 (1991).
2. Zhou, Q. Y., C. Li, M. E. Olah, G. L. Stiles, and O. Civelli. Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **89**:7432–7436 (1992).
3. Salvatore, C. A., M. A. Jacobson, H. E. Taylor, J. Linden, and R. G. Johnson. Molecular cloning and characterization of the human A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **90**:10365–10369 (1993).
4. Linden, J., H. E. Taylor, A. S. Robeva, A. L. Tucker, J. H. Stehle, S. A. Rivkees, J. S. Fink, and S. M. Reppert. Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.* **44**:524–532 (1993).
5. Hill, R. J., J. J. Oleynek, C. F. Hoth, M. A. R. Kiron, W. F. Weng, R. T.

[3] Mast cell mediators such as histamine can produce vasoconstriction or vasodilation. Microvascular vasoconstriction is mediated in part by histamine and thromboxane acting on vascular smooth muscle cell receptors (9). Systemic vasodilation and hypotension secondary to A_3 adenosine receptor activation are mediated in part by circulating histamine, which triggers nitric oxide release from endothelial cells.

Wester, W. R. Tracey, D. R. Knight, R. A. Buchholz, and S. P. Kennedy. Cloning, expression and pharmacological characterization of rabbit adenosine A(1) and A(3) receptors. *J. Pharmacol. Exp. Ther.* **280**:122–128 (1997).
6. Olah, M. E., C. Gallo-Rodriguez, K. A. Jacobson, and G. L. Stiles. ^{125}I-4-Aminobenzyl-5′-*N*-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol.* **45**:978–982 (1994).
7. Gilfillan, A. M., G. A. Wiggan, and A. F. Welton. Pertussis toxin pretreatment reveals differential effects of adenosine analogs on IgE-dependent histamine and peptidoleukotriene release from RBL-2H3 cells. *Biochim. Biophys. Acta* **1052**:467–474 (1990).
8. Ramkumar, V., G. L. Stiles, M. A. Beaven, and H. Ali. The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* **268**:16887–16890 (1993).
9. Shepherd, R. K., J. Linden, and B. R. Duling. Adenosine-induced vasoconstriction in vivo: role of the mast cell and A(3) adenosine receptor. *Circ. Res.* **78**:627–634 (1996).
10. Bjorck, T., L. E. Gustafsson, and S. E. Dahlen. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am. Rev. Respir. Dis.* **145**:1087–1091 (1992).
11. Cushley, M. J., and S. T. Holgate. Adenosine-induced bronchoconstriction in asthma: role of mast cell-mediator release. *J. Aller. Clin. Immunol.* **75**:272–278 (1985).
12. Linden, J. Cloned adenosine A_3 receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol. Sci.* **15**:298–306 (1994).
13. Caughey, G. H., S. C. Lazarus, N. F. Viro, W. M. Gold, and J. A. Nadel. Tryptase and chymase: comparison of extraction and release in two dog mastocytoma lines. *Immunology* **63**:339–344 (1988).
14. Chapman, K. R., K. Ljungholm, and A. Kallen. Long-term xanthine therapy of asthma: enprofylline and theophylline compared: International Enprofylline Study Group. *Chest* **106**:1407–1413 (1994).
15. Linden, J., A. Patel, and S. Sadek. [^{125}I]Aminobenzyladenosine, a new radioligand with improved specific binding to adenosine receptors in heart. *Circ. Res.* **56**:279–284 (1985).
16. Cullen, B. R. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* **152**:684–703 (1987).
17. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
18. Marquardt, D. M. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indust. Appl. Math.* **11**:431–441 (1963).
19. Linden, J. Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel binding by 50%. *J. Cyclic Nucleotide Res.* **8**:163–172 (1982).
20. Schwartz, L. B., K. F. Austen, and S. I. Wasserman. Immunologic release of *beta*-hexosaminidase and *beta*-glucuronidase from purified rat serosal mast cells. *J. Immunol.* **123**:1445–1450 (1979).
21. Marquardt, D. L., L. L. Walker, and S. Heinemann. Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J. Immunol.* **152**:4508–4515 (1994).
22. Motulsky, H. J., and L. A. Ransnas. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.* **1**:365–374 (1987).
23. Peachell, P. T., M. Columbo, A. Kagey-Sobotka, L. M. Lichtenstein, and G. Marone. Adenosine potentiates mediator release from human lung mast cells. *Am. Rev. Respir. Dis.* **138**:1143–1151 (1988).
24. Ali, H., O. H. Choi, P. F. Fraundorfer, K. Yamada, H. M. S. Gonzaga, and M. A. Beaven. Sustained activation of phospholipase D *via* adenosine A_3 receptors is associated with enhancement of antigen- and Ca^{++}-ionophore-induced secretion in a rat mast cell line. *J. Pharmacol. Exp. Ther.* **276**:837–845 (1996).
25. Carruthers, A. M., and J. R. Fozard. Effect of pertussis toxin treatment on the putative adenosine A_3 receptor-mediated hypotensive response in the rat. *Eur. J. Pharmacol.* **250**:185–188 (1993).
26. Brackett, L. E., and J. W. Daly. Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. *Biochem. Pharmacol.* **47**:801–814 (1994).
27. Robeva, A. S., R. Woodard, X. Jin, Z. Gao, S. Bhattacharya, H. E. Taylor, D. L. Rosin, and J. Linden. Molecular characterization of recombinant human adenosine receptors. *Drug Dev. Res.* **39**:243–252 (1996).
28. Clarke, H., M. J. Cushley, C. G. Persson, and S. T. Holgate. The protective effects of intravenous theophylline and enprofylline against histamine- and adenosine 5′-monophosphate-provoked bronchoconstriction: implications for the mechanisms of action of xanthine derivatives in asthma. *Pul. Pharmacol.* **2**:147–154 (1989).
29. Figler, R. A., S. G. Graber, M. A. Lindorfer, H. Yasuda, J. Linden, and J.C. Garrison. Reconstitution of bovine A_1 adenosine receptors in Sf9 cell membranes with recombinant G proteins of defined composition. *Mol. Pharmacol.* **50**:1587–1595 (1996).
30. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
31. Martin, P. L., R. J. Wysocki, R. J. Barrett, J. M. May, and J. Linden. Characterization of 8-(N-methylisopropyl)amino-N^6-(5′-endohydroxy-endonorbornyl)-9-methyladenine (WRC-0571), a highly potent and selective, nonxanthine antagonist of A_1 adenosine receptors. *J. Pharmacol. Exp. Ther.* **276**:490–499 (1996).
32. Yakel, J. L., R. A. Warren, S. M. Reppert, and R. A. North. Functional expression of adenosine A_{2b} receptors in *Xenopus* oocytes. *Mol. Pharmacol.* **43**:277–280 (1993).
33. Katsuyama, M., Y. Sugimoto, T. Namba, A. Irie, M. Negishi, S. Narumiya, and A. Ichikawa. Cloning and expression of a cDNA for the human prostacyclin receptor. *FEBS Lett.* **344**:74–78 (1994).
34. Hughes, P. J., S. T. Holgate, and M. K. Church. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A_2-purinoceptor mediated mechanism. *Biochem. Pharmacol.* **33**: 3847–3852 (1984).
35. Lohse, M. J., K. Maurer, H. P. Gensheimer, and U. Schwabe. Dual actions of adenosine on rat peritoneal mast cells. *Naunyn-Schmiedebergs Arch. Pharmacol.* **335**:555–560 (1987).
36. Nyce, J. W., and W. J. Metzger. DNA antisense therapy for asthma in an animal model. *Nature (Lond.)* **385**:721–725 (1997).
37. Doyle, M. P., J. Linden, and B. R. Duling. Nucleoside-induced arteriolar constriction: a mast cell-dependent response. *Am. J. Physiol.* **266**:H2042–H2050 (1994).
38. Hoffman, H. M., L. L. Walker, and D. L. Marquardt. Mast cell adenosine induced calcium mobilization via G(i3) and G(q) proteins. *Inflammation* **21**:55–68 (1997).
39. Shin, Y., J. W. Daly, and K. A. Jacobson. Activation of phosphoinositide breakdown and elevation of intracellular calcium in a rat RBL-2H3 mast cell line by adenosine analogs: involvement of A(3)-adenosine receptors. *Drug Dev. Res.* **39**:36–46 (1996).
40. Marquardt, D. L., and L. L. Walker. Alteration of mast cell responsiveness to adenosine by pertussis toxin. *Biochem. Pharmacol.* **37**:4019–4025 (1988).
41. Feoktistov, I., and I. Biaggioni. Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells: an enprofylline-sensitive mechanism with implications for asthma. *J. Clin. Invest.* **96**:1979–1986 (1995).

Send reprint requests to: Joel Linden, Ph.D., Box MR4 6012, Health Sciences Center, University of Virginia, Charlottesville, VA 22908. E-mail: jlinden@virginia.edu

Adenosine A_{2b} Receptors Evoke Interleukin-8 Secretion in Human Mast Cells

An Enprofylline-sensitive Mechanism with Implications for Asthma

Igor Feoktistov and Italo Biaggioni
Divisions of Clinical Pharmacology and Cardiology, Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, Tennessee 32732-2195

Abstract

Adenosine potentiates mast cell activation, but the receptor type and molecular mechanisms involved have not been defined. We, therefore, investigated the effects of adenosine on the human mast cell line HMC-1. Both the A_{2a} selective agonist CGS21680 and the A_{2a}/A_{2b} nonselective agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) increased cAMP, but NECA was fourfold more efficacious and had a Hill coefficient of 0.55, suggesting the presence of both A_{2a} and A_{2b} receptors. NECA 10 μM evoked IL-8 release from HMC-1, but CGS21680 10 μM had no effect. In separate studies we found that enprofylline, an antiasthmatic previously thought to lack adenosine antagonistic properties, is as effective as theophylline as an antagonist of A_{2b} receptors at concentrations achieved clinically. Both theophylline and enprofylline 300 μM completely blocked the release of IL-8 by NECA. NECA, but not CGS21680, increases inositol phosphate formation and intracellular calcium mobilization through a cholera and pertussis toxin–insensitive mechanism. In conclusion, both A_{2a} and A_{2b} receptors are present in HMC-1 cells and are coupled to adenylate cyclase. In addition, A_{2b} receptors are coupled to phospholipase C and evoke IL-8 release. This effect is blocked by theophylline and enprofylline, raising the possibility that this mechanism contributes to their antiasthmatic effects. (*J. Clin. Invest.* 1995. 96:1979–1986.) Key words: adenosine • mast cells • phospholipase C • interleukin-8 • enprofylline

Introduction

It has long been recognized that adenosine interacts with mast cells (1). Adenosine does not produce direct activation of mast cells, but it potentiates mast cell activation induced by a variety of stimuli. The adenosine receptor type present in mast cells varies depending on the type of mast cell and species studied. A3 receptors potentiate activation of rat basophil leukemia cells (RBL-2H3), a cell line used as a model for rat mast cells (2). On the other hand, mRNA for both A_{2a} and A_{2b} receptors have been identified in mouse bone marrow–derived mast cells. Activation of these cells appears to be modulated by A_{2b} receptors (3). A_2 receptors appear to mediate activation of human mast cells (4), but the receptor subtype involved is not known.

The molecular mechanisms by which adenosine potentiates mast cell activation are not fully defined. It was initially proposed that cAMP is involved in this process (5), but, at least in rat mast cells, the effect of adenosine on mediator release seems to be independent of cAMP (6). More recently, it has been shown that adenosine activates protein kinase C in mouse bone marrow–derived mast cells. Furthermore, adenosine-induced potentiation of mediator release in these cells was mimicked by low concentrations of direct activators of protein kinase C. High concentrations of these activators, however, produced the opposite effect (7). Adenosine A3 receptors are also reported to activate phospholipase C via a pertussis toxin–sensitive mechanisms (8).

Although the functional relevance of adenosine actions on mast cells remains to be established, indirect evidence suggest that adenosine activates mast cells in the human lung, provoking bronchoconstriction (9, 10). It has been proposed, therefore, that adenosine plays a role in asthma. We believe it is important to determine the adenosine receptor type(s) present in human mast cells, given the disparity found among different species, and to investigate their molecular mechanism of action.

A major limitation in this area of research has been the difficulty in obtaining a pure preparation of human mast cells. Adenosine A_2 receptors have been reputed to potentiate activation of partially purified dispersed human lung mast cells (4). Inhibition of mast cell activation by adenosine has also been reported in human lung fragments (11), dispersed human lung mast cells (12), and purified lung mast cells (4), depending on the concentration of adenosine used, or the time of incubation. The interpretation of these results, however, can be confounded by the potential indirect effects of adenosine on cell types other than mast cells present in these preparations.

A human mast cell line (HMC-1),[1] derived from a patient with mast cell leukemia, has recently been described (13). Phenotypic characterization of HMC-1 cells revealed considerable similarities with normal human mast cells. HMC-1 cells contain tryptase but not chymase. On the basis of their neutral protease contents, therefore, HMC-1 cells resemble the MC_T type of human mast cells (14), which correspond to the human lung mast cell (15). We have used this cell line to study adenosine receptors and their intracellular signaling pathways.

A major challenge to the hypothesis that adenosine-induced bronchoconstriction plays a role in asthma is the fact that enpro-

Address correspondence to Italo Biaggioni, Department of Pharmacology, AA-3228 MCN, Vanderbilt University, Nashville, TN 37232. Phone: 615-343-6499; FAX: 619-343-8649; E-mail: Biaggii@ctrvax.vanderbilt.edu

Received for publication 28 February 1995 and accepted in revised form 21 June 1995.

J. Clin. Invest.

0021-9738/95/10/1979/08 $2.00
Volume 96, October 1995, 1979–1986

1. *Abbreviations used in this paper:* CGS, 2-[*p*-(carboxyethyl)-phenylethylamino]-5'-*N* ethylcarboxamidoadenosine hydrochloride; DPSPX, 1,3-dipropyl-8-sulfophenylxanthine; G-protein, guanine nucleotide-binding regulatory protein; HEL, human erythroleukemia; HMC-1, human mast cell line; NECA, 5'-*N* ethylcarboxamidoadenosine.

fylline, a theophylline analogue, is an effective antiasthmatic but is thought to lack adenosine antagonistic properties. However, the effect of enprofylline on A_{2b} receptors has not, to our knowledge, been previously studied. We, therefore, examined the effect of enprofylline on the actions of adenosine on human erythroleukemia cells known to be mediated by A_{2b} receptors (16).

Methods

Cells. Human erythroleukemia (HEL) cells were obtained from the American Type Culture Collection (TIB 180; Rockville, MD) and maintained in suspension culture at a density between 3 and 9 × 10^5 cells/ml by dilution with RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 10% (vol/vol) newborn calf serum, antibiotics, and 2 mM glutamine.

HMC-1 cells were generous gift from doctor J. H. Butterfield (Mayo Clinic, Rochester, MN) and maintained in suspension culture at a density between 3 and 9 × 10^5 cells/ml by dilution with Iscove's medium supplemented with 10% (vol/vol) FBS, 2 mM glutamine, antibiotics, and 1.2 mM α-thioglycerol. Cells were kept under humidified atmosphere of air/CO_2 (19:1) at 37°C.

Measurement of intracellular calcium. Cytosolic free calcium concentrations were determined by fluorescent dye technique. HMC-1 cells (2 × 10^6 cells/ml) were loaded with 1 μM FURA-2/acetoxymethyl ester in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM Hepes-NaOH, pH 7.4, and 0.35% BSA. After incubation for 30 min, cells were washed to remove excess of FURA-2 and were resuspended in the same buffer containing 0.25 U/ml adenosine deaminase. HMC-1 cells were suspended at a concentration of 10^5 cells/ml in the same buffer without BSA. Fluorescence was monitored at an emission wavelength of 510 mm and excitation wavelengths of 340 and 380 mm. Maximal fluorescence was determined by addition of 20 μl of 0.4% digitonin. Minimal fluorescence was determined by addition of 40 μl of 1 M EGTA. The intracellular calcium was calculated using previously described formulas (17), assuming a K_d of 224 nM. Fluorescence was measured with a spectrofluorimeter (Fluorolog 2; Spex Industries, Inc., Edison, NJ) in a thermostated cuvette (37°C).

Measurement of cAMP. Before each experiment, HMC-1 and HEL cells were harvested, washed by centrifugation (100 *g* for 10 min), and resuspended in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM Hepes-NaOH, pH 7.4, and 0.25 U/ml adenosine deaminase to a concentration of 3 × 10^6 cells/ml (HMC-1 cells) or 10^7 cells/ml (HEL cells). HMC-1 cells were preincubated for 3 min at 37°C in a total vol of 198 μl (178 μl for HEL cells) of buffer, containing the cAMP phosphodiesterase inhibitor papaverine (0.1 mM). cAMP accumulation in response to adenosine agonists was measured by the addition of the agonist (2 μl) to the cell suspension. The adenosine antagonists enprofylline and theophylline (20 μl), or buffer control, were added to HEL cells as indicated. Cells were then mixed with a vortex and the incubation allowed to proceed for 3 min (2 min for HEL cells) at 37°C. The reaction was stopped by addition of 50 μl of 25% TCA. TCA-treated extracts were washed five times with 10 vol of water-saturated ether. cAMP concentrations were determined by competition binding of tritium-labeled cAMP to a protein derived from bovine muscle which has high specificity for cAMP (18) (cAMP assay kit, TRK.432; Amersham Corp., Arlington Heights, IL).

Measurement of [^{3}H]inositol phosphate formation. Formation of inositol phosphate was determined using a modification of the procedure described by K. Seuwen et al. (19). HMC-1 cells, at the concentration 10^7 cells/ml, were labeled to equilibrium with *myo*-[^{3}H]inositol (2 μCi/ml, DuPont-NEN, Boston, MA) for 24 h in serum-free Iscove's medium containing 0.25 U/ml adenosine deaminase. The HMC-1 cells were then washed twice and resuspended in buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM Hepes-NaOH, pH 7.4, 1 U/ml adenosine deaminase, and 20 mM LiCl to a concentration of 5 × 10^7 cells/ml. After preincubation at room temperature for 10 min, cells (178 μl) were added to tubes containing adenosine agonists (2 μl) and antagonists (20 μl) or their corresponding vehicles, and the incubation was allowed to proceed for 30 min at 37°C. Cells were collected by centrifugation and resuspended in 200 μl of ice-cold 10 mM formic acid (pH 3). After 30 min, this solution, containing the extracted inositol phosphates and inositol, was collected by centrifugation and diluted with 800 μl of 5 mM NH_3 solution (final pH, 8–9). This solution was then applied to a column containing 0.2 ml anion exchange resin (AG 1-X8, formate form, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA). Free inositol and glycerophosphoinositol were eluted with 1.25 ml of H_2O and 1 ml of 40 mM ammonium formate/formic acid, pH 5, respectively. Total inositol phosphates were eluted in the single step with 1 ml of 2 M ammonium formate/formic acid, pH 5, and radioactivity was measured by liquid scintillation counting.

Determination of IL-8 secretion. HMC-1 cells were harvested and resuspended to a concentration of 10^6 cells/ml in serum-free Iscove's media, containing 0.25 U/ml adenosine deaminase. Cells were incubated for 18 h under humidified atmosphere of air/CO_2 (19:1) at 37°C with the reagents indicated in Results. At the end of this incubation period the culture media were collected by centrifugation at 100 *g* for 10 min. IL-8 concentrations were measured in the culture media using an ELISA method (Quantikine; R & D Systems, Minneapolis, MN).

Drugs. 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), 5′-*N*-ethylcarboxamidoadenosine (NECA), 2-[*p*-(carboxyethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21,680) and 3-*n*-propylxanthine (enprofylline) were purchased from Research Biochemicals, Inc. (Natick, MA). Papaverine, calcium ionophore A23187, PMA, and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO). Forskolin was purchased from Calbiochem Corp. (La Jolla, CA).

Data analysis. Calculation of 50% effective concentration (EC_{50}) values from dose-response curves was performed by nonlinear regression analysis using InPlot 4.0 software (GraphPAD Software for Science, San Diego, CA) on a microcomputer. Statistical analysis was performed using InStat 2.0 software (GraphPAD Software). Unpaired Student's *t* test was used for single comparisons. The criterion for significance was $P < 0.05$. Results are presented as mean±standard error.

Results

Effect of enprofylline on adenosine A_{2b} receptors in human erythroleukemia cells. Increasing concentrations of enprofylline produced parallel rightward shifts of the dose-response curve for NECA-induced cAMP accumulation (Fig. 1 *A*). Schild regression analysis revealed slopes close to unity (0.9), indicating that enprofylline acts as a simple competitive antagonist of A_{2b} receptors. The intercept of this linear regression, which is used to estimate the K_i of antagonists, was 7 μM (Fig. 1 *B*). We compared the effects of enprofylline to those of theophylline, another antiasthmatic agent, and DPSPX. Schild analysis of these compounds yielded slopes of 0.9 and 1, and K_i of 13 μM and 141 nM, respectively.

Effect of adenosine agonists on IL-8 production in human mast cells. Incubation of HMC-1 cells with a combination of 50 ng/ml PMA and 200 nM calcium ionophore A23187 for 18 h increased IL-8 release from 12±4 to 1,785±86 pg/10^6 cells ($n = 5$, $P < 0.001$). The response to this combination of drugs was decreased by 22% in the presence of 300 μM enprofylline (to 1,391±74 pg/10^6 cells, $n = 5$, $P < 0.01$). Incubation with enprofylline alone had no significant effect on spontaneous release of IL-8 (to 8±6 pg/10^6 cells, $n = 5$, $P > 0.5$).

Incubation of HMC-1 cells with the nonselective A_{2b}/A_{2b}

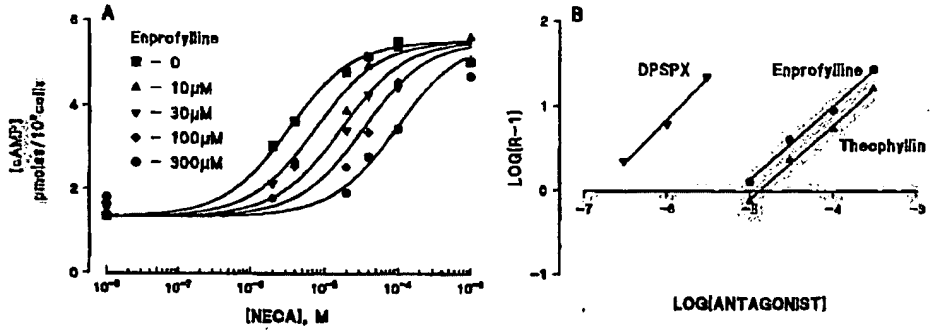


Figure 1. Antagonistic effects of methylxanthines on A_{2b} receptors in HEL cells. (*A*) Dose-response curves for accumulation of cAMP produced by NECA in HEL cells. Dose-response curves were repeated in the absence and in presence of increasing concentrations of enprofylline, which produced a progressive shift to the right. A representative experiment of four is shown. (*B*) Schild analysis of the data from (*A*) and data obtained in similar experiments with the adenosine receptor antagonists theophylline and DPSPX. Schild analysis revealed linear relationships for all compounds, implying competitive antagonism at A_{2b} receptors.

agonist NECA (10 μM) for 18 h resulted in a 26-fold increase in the release of IL-8 (from 12±4 to 306±23 pg/10^6 cells, n = 5, $P < 0.001$). The increase in IL-8 release produced by NECA was blocked if cells were incubated in the presence of 300 μM enprofylline (38±6 pg IL-8/10^6 cells, $n = 5$, $P < 0.001$ compared to NECA + vehicle) or 300 μM theophylline (57±10 pg IL-8/10^6 cells, n = 5, $P < 0.001$ compared to NECA + vehicle) (Fig. 2 *A*). In contrast to the stimulatory effects of NECA on IL-8 release, the selective A_{2b} agonist CGS 21680 (10 μM) produced only a marginal increase in IL-8 release (29±4 pg IL-8/10^6 cells, n = 5, P = 0.05) comparable to the effect produced by NECA in the presence of enprofylline.

We also determined if adenosine potentiated IL-8 production induced by an independent stimulus. PMA (0.6 ng/ml, 1 nM) stimulated IL-8 production to 525±20 pg/10^6 cells (n = 3). NECA (10 μM) increased IL-8 production to 356±20 pg/10^6 cells (n = 3) (Fig. 2 *B*). The combination of NECA and PMA stimulated IL-8 production to 2,594±122 pg/10^6 cells (n = 3). To determine if this potentiation could be due to activation of adenylate cyclase by NECA, we used forskolin and 8-Br-cAMP as controls. Neither forskolin (1, 10, and 100 μM, shown to stimulate adenylate cyclase in these cells) nor 8-Br-cAMP (1, 10, and 100 μM) induced IL-8 production. Likewise, these compounds had no effect on PMA-induced IL-8 production (data not shown).

Effect of adenosine receptor activation on cAMP in HMC-1 cells. The unstimulated level of cAMP in HMC-1 was 2.8±0.1 pmol/10^6 cells. Forskolin (100 μM) increased cAMP accumulation 14-fold, to 38.4±3.0 pmol/10^6 cells (n = 6, $P < 0.001$). Forskolin-stimulated cAMP accumulation was not affected by coincubation with 100 μM CGS 21680 (38.0±5.1 pmoles/10^6 cells, n = 3, $P > 0.05$ compared to forskolin + vehicle) but was greater in the presence of 100 μM NECA (to 56.37±5.1 pmol/10^6 cells, n = 3, $P < 0.001$ compared to forskolin + vehicle).

Adenosine agonists produced a dose-dependent accumulation of cAMP in HMC-1 in the absence of forskolin (Fig. 3). NECA was more efficacious than CGS 21680; at concentrations producing maximal effects (1 mM), NECA produced an eight-fold increase in cAMP (to 22.3±3.2 pmol/10^6 cells, n = 3),

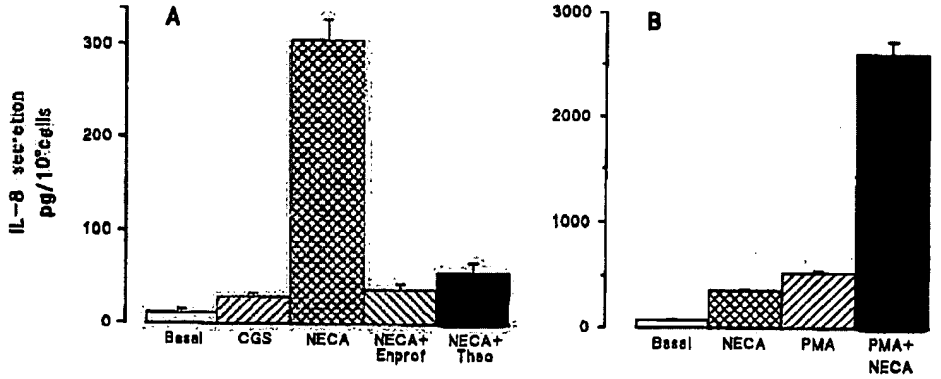


Figure 2. Release of IL-8 from HMC-1 cells. (*A*) Release of interleukin-8 into culture media by unstimulated HMC-1 cells (*Basal*), or by cells stimulated with 10 μM CGS 21,680 (*CGS*), and with 10 μM NECA in the absence (*NECA*) or in the presence of 300 μM enprofylline (*NECA + Enprof*), or 300 μM theophylline (*NECA + Theo*). Values are expressed as mean±standard error of five experiments. (*B*) Release of IL-8 into culture media by unstimulated HMC-1 cells (*Basal*), or by cells stimulated with 10 μM NECA, 1 nM PMA, or 10 μM NECA combined with 1 nM PMA. Values are expressed as mean±standard error of three experiments. Note the difference in the scale of the y-axis between panels *A* and *B*.

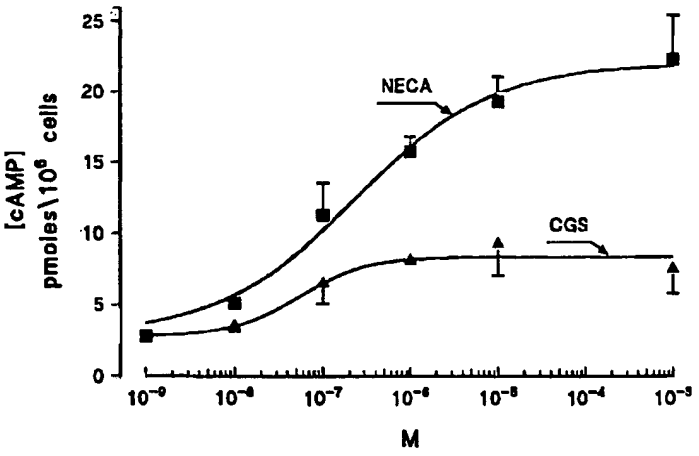


Figure 3. Effect of increasing concentrations of adenosine receptor agonists on cAMP accumulation in HMC-1 cells. Values are expressed as mean±standard error of three experiments.

but CGS 21680 produced only a twofold increase in cAMP (to 7.5±1.7 pmol/10^6 cells, $n = 3$). Nonlinear regression analysis of concentration-response curves revealed an EC_{50} of 225 nM for NECA and 54 nM for CGS 21680. The Hill coefficient for CGS 21680 was close to unity (1.1), consistent with stimulation of cAMP production through a single adenosine receptor subtype. On the other hand, the concentration-response relationship for NECA was characterized by a Hill coefficient of 0.55, suggesting the involvement of more than one adenosine receptor subtype on this effect.

Effect of adenosine receptor activation on intracellular Ca^{2+} in HMC-1 cells. Adenosine analogues produced a dose-dependent increase in intracellular calcium content in HMC-1 (Fig. 4). NECA was more efficacious than CGS 21680; at concentrations (100 μM) producing maximal effects, NECA and CGS 21680 increased Ca^{2+} by 210±6 nM and 75±6 nM, respectively. On the other hand, both agonists had similar potencies; the EC_{50} for NECA and CGS 21680, estimated by nonlinear analysis, were 334 and 296 nM, respectively. It is worth noting, however, that NECA produced a shallow concentration-response curve, with a Hill coefficient significantly lower than unity (0.6). This suggests that NECA stimulates intracellular Ca^{2+} through an interaction with more than one receptor site.

In ancillary studies we demonstrated that the NECA-induced increase in FURA-2 fluorescence could not be explained by leakage of the dye to the extracellular space, because we found that 100 μM NECA did not increase FURA-2 content in the supernatant (data not shown). The increase in intracellular Ca^{2+} produced by NECA, therefore, can be explained either by an increase in extracellular Ca^{2+} influx or by mobilization of internal stores. NECA (10 μM) increased intracellular Ca^{2+} even in cells incubated in the absence of extracellular Ca^{2+}, that is, in a calcium-free medium containing 1 mM EGTA (Fig. 5 *A*). This indicates that NECA increases intracellular Ca^{2+} by evoking Ca^{2+} mobilization. On the other hand, CGS 21680 had no effect on intracellular calcium under these conditions (Fig. 5 *A*). It should be noted that NECA induced a sustained elevation in Ca^{2+} levels in the presence of extracellular Ca^{2+}, but, in the absence of extracellular Ca^{2+}, it induced a transient rise in cytoplasmic Ca^{2+}.

We used an additional approach to determine if NECA can stimulate extracellular calcium influxes or intracellular calcium

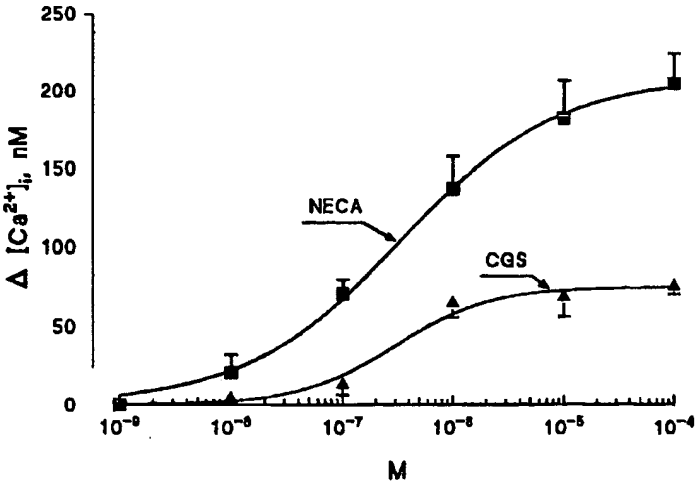
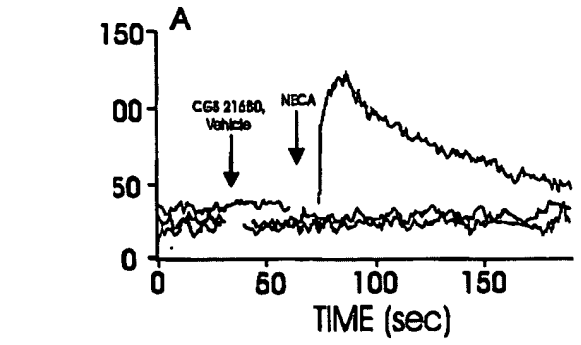
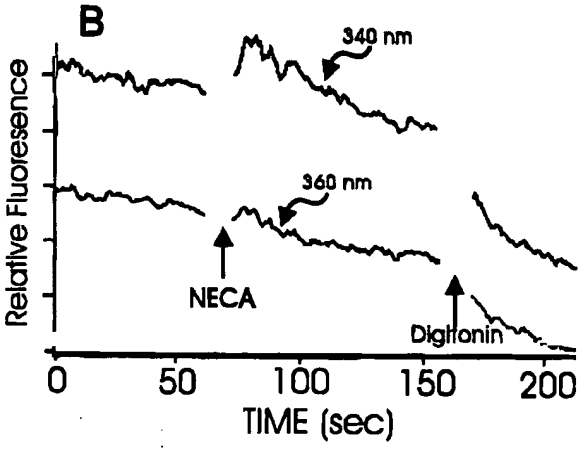
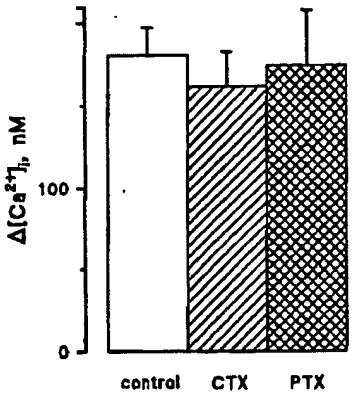


Figure 4. Effect of increasing concentrations of adenosine receptor agonists on free intracellular Ca^{2+} levels in HMC-1 cells. Experiments were performed in the presence of 1 mM $CaCl_2$ in the extracellular media. Values are expressed as mean±standard error of six experiments.



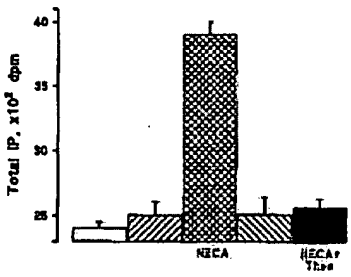




mobilization in HMC-1 cells. Mn^{2+} and Ca^{2+} have been shown to share the same channel for entry into cells. At an excitation wavelength of 360 nm and an emission wavelength of 500 nm, fluorescence is selectively quenched by influx of Mn^{2+} and is unaltered by changes in Ca^{2+}. On the other hand, mobilization of Ca^{2+} from internal stores can be assessed simultaneously by monitoring fluorescence at an excitation wavelength 340 nm in cells incubated in a calcium-free medium (20). For these studies Mn^{2+} was added into Ca^{2+}-free buffer just before each measurement, to a final concentration of 100 μM. As shown in Fig. 5 *B*, the addition of NECA to the incubation medium produced an initial increase in the Ca^{2+} signal, followed by slight quenching of the FURA 2 signal by Mn^{2+}. This suggests that NECA induces an initial mobilization of Ca^{2+} from intracellular stores and a subsequent (and probably calcium-mediated) cation influx from extracellular media.

We then determined the potential role of G-proteins in adenosine actions on intracellular Ca^{2+} in HMC-1 cells. Cholera toxin was used as a way to increase the basal activity of the stimulatory guanine nucleotide-binding proteins (G_s) and pertussis toxin was used as a way to block the family of inhibitory guanine nucleotide-binding protein (G_i). Pretreatment of HMC-1 cells with 100 ng/ml cholera toxin or 500 ng/ml pertussis toxin for 24 h had no effect on basal or NECA-stimulated intracellular Ca^{2+} levels (Fig. 6). This effect, therefore, is not mediated by coupling to G_s or G_i proteins. To determine the potential role of cAMP we used forskolin. Forskolin, at concentrations of 10 and 100 μM, had no effect on basal Ca^{2+} or on the increase in Ca^{2+} produced by NECA (data not shown). Forskolin effectively stimulated adenylate cyclase at these concentrations. These results, therefore, suggest that NECA-induced rise in intracellular Ca^{2+} is not mediated by an increase cAMP levels in HMC-1.

Effect of adenosine receptor activation on inositol phosphate formation in HMC-1 cells. The major pathway of intracellular calcium mobilization involves phospholipase C activation with phosphoinositide hydrolysis. To determine the role of this pathway on adenosine actions, we measured the accumulation of total inositol phosphates in the presence of 20 mM LiCl. NECA 10 μM considerably increased the accumulation of inositol phosphates (from 2,402±48 dpm/tube to 3,906±99 dpm/tube, $n = 5$, $P < 0.001$, Fig. 7). In contrast, 10 μM CGS 21680 had no effect on levels of inositol phosphates. Enprofylline 300 μM and theophylline 300 μM blocked the increase in inositol phosphates produced by NECA in HMC-1 cells. Neither antagonist affected basal levels of inositol phosphates.



Discussion

The hypothesis that adenosine plays a role in asthma was initially suggested by the recognition that theophylline blocks adenosine receptors at concentrations achieved clinically (21, 22). Even though methylxanthines may have other mechanisms of action in vitro, such as inhibition of phosphodiesterases or mobilization of intracellular calcium, it is believed that these effects require higher concentrations than those clinically achieved in vivo (21). The antiasthmatic effects of theophylline, therefore, could result from blockade of endogenous adenosine. This assumes, however, that adenosine is able to provoke asthma. In support of this assumption, administration by inhalation of adenosine, or of its precursor AMP, provokes bronchoconstriction in asthmatics but not in normal subjects (23). Adenosine-induced bronchoconstriction is most likely mediated by activation of mast cells because this effect is blocked not only by adenosine receptor antagonists (9) but also by selective histamine H1 blockers (24, 25) and cromolyn sodium (23, 26). These in vivo findings are in agreement with the observations made by Marquardt and colleagues demonstrating that adenosine activates mast cells in vitro (7, 27).

A major challenge to the hypothesis that adenosine contributes to asthma comes from the "enprofylline paradox." Enprofylline (3-*N*-propylxanthine) is as effective as theophylline (1, 3, 7, trimethylxanthine) in the treatment of asthma, but was, heretofore, believed not to block adenosine receptors (28, 29). This assertion, however, derives mostly from studies performed before the recognition of the existence of A_2 receptor subtypes. More recently, it has been shown that enprofylline does not compete for A_3 receptor binding (30). To the best of our knowledge, the possibility that enprofylline blocks A_{2b} receptor has been overlooked. Our studies found enprofylline to be as potent as theophylline as an A_{2b} receptor antagonist. Furthermore, the K_i of enprofylline (7 μM) is within its recommended therapeutic plasma levels (5–25 μM), indicating that plasma concentrations reached under clinical conditions are sufficient to block A_{2b} receptors.

It has been emphasized that A_{2b} receptors have, in general, significantly less affinity for adenosine agonists than do the other known adenosine receptor subtypes. This is indeed a criterion used to characterize A_{2b} receptors. Less recognized is the fact that A_{2b} receptors may have a similar or even greater affinity to some adenosine antagonists. For example, the affinity of DPSPX for A_{2b} receptors (K_i, 0.1 μM) is 10-fold higher than that for A_{2a} receptors (16). DPSPX is, to the best of our knowledge, the most potent antagonist of A_{2b} receptors known to date, but a systematic search for methylxanthine analogues with A_{2b} blocking properties has not been undertaken. It is possible that more potent antagonists exist or can be developed. Even though enprofylline is 100-fold less potent than DPSPX, it is the only known selective A_{2b} antagonist to date. Therefore, it can become a useful pharmacological tool to characterize A_{2b} receptors and their functional relevance.

It has been assumed that enprofylline alleviates asthma through mechanisms other than adenosine receptor antagonism. Intravenous enprofylline was found to be less effective than theophylline in blocking adenosine-induced bronchoconstriction (31). Plasma concentrations of enprofylline, however, were four times lower than theophylline in that study, whereas our results suggest that both methylxanthines are equipotent in blocking A_{2b} receptors. We believe, therefore, that our findings

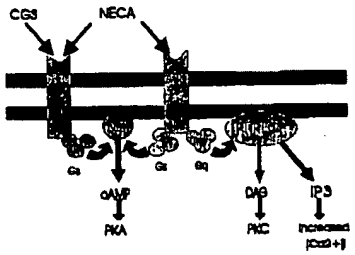


Figure 8. Proposed intracellular pathways for adenosine receptors in HMC-1 cells. See text for details. *CGS*, CGS 21680; *AC*, adenylate cyclase; *PI-PLC*, phosphatidylinositol specific phospholipase C; *PKA*, cAMP-dependent protein kinase; *DAG*, diacylglycerol; *IP3*, inositol trisphosphate; *PKC*, protein kinase C.

solve the enprofylline paradox and raise the possibility that its antiasthmatic effects are due, at least partially, to blockade of adenosine A_{2b} receptors. This postulate, however, assumes that A_{2b} receptors activate mast cells. It is this possibility that we wanted to examine in the human mast cell line HMC-1.

Our results indicate the presence of both A_{2a} and A_{2b} receptors in HMC-1 cells. Both receptor subtypes contribute to adenylate cyclase activation, most likely through coupling with G_s proteins, as previously described in other cell types (16). It is possible that A_{2a} receptors also contribute to the increase in intracellular calcium observed in HMC-1 cells. This would explain the small increase in intracellular calcium produced by CGS 21680 (Fig. 4). This effect, however, is not observed in cells incubated in the absence of extracellular calcium. We found no functional consequence of this phenomenon, since we have no evidence that A_{2a} receptors contribute to phospholipase C activation or IL-8 release in HMC-1 cells. For these reasons, we have not explored the molecular mechanisms of A_{2a}-mediated calcium rise. Because forskolin had no effect on intracellular calcium, it is unlikely that this effect is mediated by cAMP. It is possible that A_{2a} and A_{2b} receptors are directly coupled to a calcium channel through G_s proteins, as suggested for A_{2b} receptors (32) and other G_s-coupled receptors (33, 34).

One of the main differences between A_{2a} and A_{2b} receptors entails modulation of intracellular calcium. Whereas A_{2a} receptors generally inhibit intracellular calcium rises in most cell types (35, 36), activation of A_{2b} receptors generally potentiate intracellular calcium rises. The molecular mechanisms by which A_{2b} modulates intracellular calcium appear to be different among cell types. A_{2b} receptors potentiate a P-type calcium current in hippocampal neurons (37). In human erythroleukemia cells, A_{2b} receptors facilitate calcium influx through a G_s protein–coupled, but cAMP-independent process (32), most likely involving the opening of a calcium channel. Our results show that A_{2b} receptors increase intracellular calcium in HMC-1 cells through a cholera- and pertussis toxin–insensitive process. These findings suggest that a guanine nucleotide-binding protein of the G_q family is involved. The increase in phosphoinositide hydrolysis and intracellular calcium mobilization suggest that A_{2b} receptors activate phospholipase C, and presumably also protein kinase C (Fig. 8).

IL-8 secretion from HMC-1 cells requires increases in gene transcription and de novo protein synthesis (38), but the cellular events leading to this process have not been characterized. Our results do not support involvement of adenylate cyclase, since neither forskolin nor 8-Br-cAMP stimulated IL-8 production. It could be proposed that adenosine-induced IL-8 secretion is the

result of A_{2b}-mediated phospholipase C activation. This would lead to phosphoinositide hydrolysis, calcium mobilization, and protein kinase C activation. In support of this proposal, the most potent stimulus known to evoke IL-8 release from HMC-1 is activation of protein kinase C by phorbol ester (38). Furthermore, there is a striking similarity between the effects of adenosine agonists and antagonists on inositol phosphate formation and IL-8 release (compare Figs. 2 and 7). A direct causal relationship, however, has not been proven.

Marquardt and colleagues (7) have previously demonstrated that adenosine analogues induce translocation of protein kinase C activity in cell membranes of mouse bone marrow–derived mast cells, and suggested that this process contributes to adenosine-induced potentiation of mast cell activation. More recently, these investigators have reported that A_{2b} receptors mediate adenosine actions in these mast cells (3). Our results in human mast cells, therefore, correspond closely to those found by Marquardt and colleagues, in mouse bone marrow–derived mast cells. Two differences between their finding and ours are worth noting. First, adenosine actions on mouse bone marrow–derived mast cells were found to be pertussis toxin–sensitive (39) whereas we found no evidence of G_i coupling in these human mast cells. Second, adenosine does not activate bone marrow–derived mast cells directly; it rather potentiates mast cell activation. In contrast, adenosine alone produced significant IL-8 release in HMC-1 cells. Adenosine also greatly potentiated IL-8 production induced by PMA (Fig. 2 *B*). This effect appears to be synergistic rather than additive and is similar to that observed in mouse bone marrow–derived mast cells. Adenylate cyclase is not involved in this process, since potentiation of PMA-induced IL-8 production was not reproduced by 8-Br-cAMP. Further studies are required to define the mechanisms of adenosine-induced potentiation. It should be noted that inhaled adenosine does not require other stimulants to provoke bronchoconstriction in asthmatics. Whether mast cells are in a constant "preactivated" state in asthma is speculative.

It has been suggested that the recently recognized A3 receptor modulates mast cell activation (40). mRNA encoding A3 receptors is expressed in rat basophil leukemia cells (RBL-2H3), and these receptor types reportedly potentiate activation of these surrogate rat mast cells (2). However, A3 receptors, while prominent in rat mast cells, have not been shown to be functionally present in mast cells derived from other species. Also, the rat A3 receptor is generally insensitive to methylxanthines, including theophylline (41). Human (42) and sheep (30) A3 receptors are sensitive to the antagonistic effects of theophylline and other methylxanthines, but they have a low affinity to enprofylline (30). A3 receptors, therefore, are less likely to be involved in asthma, given the efficacy of enprofylline in the treatment of this disease process. Although we found no evidence for the functional expression of A3 receptors in HMC-1 cells, it remains possible that this receptor type is expressed in other human mast cells.

In summary, our results indicate that the human mast cell line HMC-1 functionally expresses A_{2b} receptors. Their activation leads to increases in phosphoinositide hydrolysis, intracellular calcium mobilization, and IL-8 secretion. Enprofylline is a competitive antagonist of A_{2b} receptors and inhibits adenosine-mediated IL-8 secretion in human mast cells. Taken together, these results support the hypothesis that A_{2b} receptors are involved in the putative role of adenosine in asthma. This conclusion, however, is based on the assumptions that antagonism of A_{2b} receptors accounts for the antiasthmatic effects of enprofylline, and that the HMC-1 cell line is an adequate model for adenosine receptors in human lung mast cells. The validity of these assumptions remains to be determined.

Acknowledgments

The authors thank Drs. William Serafin and Jack Wells for helpful suggestions in the design of studies and interpretation of results, and Dr. J. H. Butterfield for providing HMC-1 cells.

This work was supported by grants RR00095 (Clinical Research Center) and HL-14192 (Specialized Center of Research in Hypertension) from the National Institutes of Health. Dr. Feoktistov is a recipient of an American Lung Association research grant.

References

1. Marquardt, D. L., C. W. Parker, and T. J. Sullivan. 1978. Potentiation of mast cell mediator release by adenosine. *J. Immunol.* 120:871–878.

2. Ramkumar, V., G. L. Stiles, M. A. Beaven, and H. Ali. 1993. The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* 268:16887–16890.

3. Marquardt, D. L., L. L. Walker, and S. Heinemann. 1994. Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J. Immunol.* 152:4508–4515.

4. Peachell, P. T., L. M. Lichtenstein, and R. P. Schleimer. 1991. Differential regulation of human basophil and lung mast cell function by adenosine. *J. Pharmacol. Exp. Ther.* 256:717–726.

5. Holgate, S. T., R. A. Lewis, and K. F. Austen. 1980. Role of adenylate cyclase in immunologic release of mediators from rat mast cells: agonist and antagonist effects of purine- and ribose-modified adenosine analogs. *Proc. Natl. Acad. Sci. USA.* 77:6800–6804.

6. Church, M. K., P. J. Hughes, and C. J. Vardey. 1986. Studies on the receptor mediating cyclic AMP-independent enhancement by adenosine of IgE-dependent mediator release from rat mast cells. *Br. J. Pharmacol.* 87:233–242.

7. Marquardt, D. L., and L. L. Walker. 1990. Modulation of mast cell responses to adenosine by agents that alter protein kinase C activity. *Biochem. Pharmacol.* 39:1929–1934.

8. Ali, H., J. R. Cunha-Melo, W. F. Saul, and M. A. Beaven. 1990. Activation of phospholipase C via adenosine receptors provides synergistic signals for secretion in antigen-stimulated RBL-2H3 cells. Evidence for a novel adenosine receptor. *J. Biol. Chem.* 265:745–753.

9. Mann, J. S., and S. T. Holgate. 1985. Specific antagonism of adenosine-induced bronchoconstriction in asthma by oral theophylline. *Br. J. Clin. Pharmacol.* 19:685–692.

10. Cushley, M. J., and S. T. Holgate. 1985. Adenosine-induced bronchoconstriction in asthma: role of mast cell-mediator release. *J. Allergy Clin. Immunol.* 75:272–278.

11. Hillyard, P. A., A. T. Nials, I. F. Skidmore, and C. J. Vardey. 1995. Characterization of the adenosine receptor responsible for the inhibition of histamine and SRS-A release from human lung fragments. *Br. J. Pharmacol.* 83:337–345.

12. Hughes, P. J., S. T. Holgate, and M. K. Church. 1984. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A2-purinoceptor mediated mechanism. *Biochem. Pharmacol.* 33:3847–3852.

13. Butterfield, J. H., D. Weiler, G. Dewald, and G. J. Gleich. 1988. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk. Res.* 12:345–355.

14. Nilsson, G., T. Blom, M. Kusche-Gullberg, I. Kjellen, J. H. Butterfield, C. Sundstrom, K. Nilsson, and L. Hellman. 1994. Phenotypic characterization of the human mast-cell line HMC-1. *Scand. J. Immunol.* 39:489–498.

15. Irani, A. A., N. M. Schechter, S. S. Craig, G. DeBlois, and L. B. Schwartz. 1986. Two types of human mast cells that have distinct neutral protease compositions. *Proc. Natl. Acad. Sci. USA.* 83:4464–4468.

16. Feoktistov, I., and I. Biaggioni. 1993. Characterization of adenosine receptors in human erythroleukemia cells. Further evidence for heterogeneity of adenosine A_2 receptors. *Mol. Pharmacol.* 43:909–914.

17. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

18. Gilman, A. G. 1970. A protein binding assay for adenosine 3′,5′-monophosphate. *Proc. Natl. Acad. Sci. USA.* 67:305–312.

19. Seuwen, K., A. Lagarde, and J. Pouyssegur. 1988. Deregulation of hamster fibroblast proliferation by mutated ras oncogenes is not mediated by constitutive

activation of phosphoinositide-specific phospholipase C. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:161–168.

20. Merritt, J. E., R. Jacob, and T. J. Hallam. 1989. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J. Biol. Chem.* 264:1522–1527.

21. Rall, T. W. 1982. Evolution of the mechanisms of action of methylxanthines: from calcium mobilizers to antagonists of adenosine receptors. *Pharmacologist.* 24:277–287.

22. Biaggioni, I., S. Paul, A. Puckett, and C. Arzubiaga. 1991. Caffeine and theophylline as adenosine receptor antagonists in humans. *J. Pharmacol. Exp. Ther.* 258:588–593.

23. Cushley, M. J., A. E. Tattersfield, and S. T. Holgate. 1984. Adenosine-induced bronchoconstriction in asthma. *Am. Rev. Respir. Dis.* 129:380–384.

24. Björck, T., L. E. Gustafsson, and S. E. Dahlén. 1992. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am. Rev. Respir. Dis.* 145:1087–1091.

25. Rafferty, P., C. R. Beasley, and S. T. Holgate. 1987. The contribution of histamine to immediate bronchoconstriction provoked by inhaled allergen and adenosine-5′monophosphate in atopic asthma. *Am. Rev. Respir. Dis.* 136:369–373.

26. Crimi, E., V. Brusasco, M. Brancatisano, E. Losurdo, and P. Crimi. 1986. Adenosine-induced bronchoconstriction: premedication with chlorpheniramine and nedocromil sodium. *Eur. J. Respir. Dis.* 69:255–257.

27. Marquardt, D. L., L. L. Walker, and S. I. Wasserman. 1984. Adenosine receptors on mouse bone marrow-derived mast cells: functional significance and regulation by aminophylline. *J. Immunol.* 133:932–937.

28. Persson, C. G. 1983. The profile of action of enprofylline, or why adenosine antagonism seems less desirable with xanthine antiasthmatics. (Review). *Agents Actions.* 13:115–129.

29. Persson, C. G. A., K. Andersson, and G. Kjellin. 1986. Effects of enprofylline and theophylline may show the role of adenosine. *Life Sci.* 38:1057–1072.

30. Linden, J., H. E. Taylor, A. S. Robeva, A. L. Tucker, J. H. Stehle, S. A. Rivkees, J. S. Fink, and S. M. Reppert. 1993. Molecular cloning and functional expression of a sheep A3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.* 44:524–532.

31. Clarke, H., M. J. Cushley, C. G. Persson, and S. T. Holgate. 1989. The protective effects of intravenous theophylline and enprofylline against histamine- and adenosine 5′monophosphate-provoked bronchoconstriction: implications for the mechanisms of action of xanthine derivatives in asthma. *Pulm. Pharmacol.* 2:147–154.

32. Feoktistov, I., J. J. Murray, and I. Biaggioni. 1994. Positive modulation of intracellular Ca^{2+} levels by adenosine A2b receptors, prostacyclin, and prostaglandin E1 via a cholera toxin-sensitive mechanism in human erythroleukemia cells. *Mol. Pharmacol.* 45:1160–1167.

33. Hamilton, S. L., J. Codinas, M. J. Hawkes, A. Yatani, T. Sawada, F. M. Strickland, S. C. Froehner, A. M. Spiegel, L. Toro, E. Stefani, et al. 1991. Evidence for direct interaction of $G_{\alpha s}$ with the Ca^{2+} channel of skeletal muscle. *J. Biol. Chem.* 266:19528–19535.

34. Scamps, F., V. Rybin, M. Pucéat, V. Tkachuk, and G. Vassort. 1992. A G_i protein couples P_2-purinergic stimulation to cardiac Ca channels without cyclic AMP production. *J. Gen. Physiol.* 100:675–701.

35. Bradley, A. B., and K. G. Morgan. 1985. Cellular Ca^{2+} monitored by aequorin in adenosine-mediated smooth muscle relaxation. *Am. J. Physiol.* 248:H109–H117.

36. Feoktistov, I., S. Paul, A. S. Hollister, D. Robertson, and I. Biaggioni. 1992. Role of cyclic AMP in adenosine inhibition of intracellular calcium rise in human platelets. Comparison of adenosine effects of thrombin-induced and epinephrine-induced platelet stimulation. *Am. J. Hypertens.* 5:147S–153S.

37. Mogul, D. J., M. E. Adams, and A. P. Fox. 1993. Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca^{2+} current in hippocampal CA3 neurons. *Neuron.* 10:327–334.

38. Selvan, R. S., J. H. Butterfield, and M. S. Krangel. 1994. Expression of multiple chemokine genes by a human mast cell leukemia. *J. Biol. Chem.* 269:13893–13898.

39. Marquardt, D. L., and L. L. Walker. 1988. Alteration of mast cell responsiveness to adenosine by pertussis toxin. *Biochem. Pharmacol.* 37:4019–4025.

40. Beaven, M. A., V. Ramkumar, and H. Ali. 1994. Adenosine A3 receptors in mast cells. *Trends Pharmacol. Sci.* 15:13–14.

41. Zhou, Q., C. Li, M. E. Olah, R. A. Johnson, G. L. Stiles, and O. Civelli. 1992. Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc. Natl. Acad. Sci. USA.* 89:7432–7436.

42. Salvatore, C. A., M. A. Jacobson, H. E. Taylor, J. Linden, and R. G. Johnson. 1993. Molecular cloning and characterization of the human A3 adenosine receptor. *Proc. Natl. Acad. Sci. USA.* 90:10365–10369.

0031-6997/97/4904-0381$03.00/0
PHARMACOLOGICAL REVIEWS
Vol. 49, No. 4
Printed in U.S.A.

Adenosine A_{2B} Receptors

IGOR FEOKTISTOV AND ITALO BIAGGIONI[a]

Divisions of Clinical Pharmacology and Cardiology, Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, Tennessee

I. Introduction

Adenosine is an endogenous nucleoside that modulates many physiological processes. Its actions are mediated by interaction with specific cell membrane receptors. Four subtypes of adenosine receptors have been cloned: A_1, A_{2A}, A_{2B}, and A_3. Significant advancement has been made in the understanding of the molecular pharmacology and physiological relevance of adenosine receptors, but our knowledge of A_{2B} receptors lags behind that of other receptor subtypes. The lack of selective pharmacological probes has hindered research in this area. Perhaps because of their lower affinity for adenosine compared with other receptors, it is often assumed that A_{2B} receptors are a low-affinity version of the A_{2A} receptor and are of lesser physiological relevance. It has been only recently that potentially important functions have been discovered for the A_{2B} receptor, prompting a renewed interest in this receptor type. It is also recently recognized that A_{2B} receptors are coupled to intracellular pathways different from those of A_{2A} receptors, a finding that may provide the basis for their distinct physiological role. A_{2B} receptors have been implicated in mast cell activation and asthma, vasodilation, regulation of cell growth, intestinal function, and modulation of neurosecretion. We try to review the recent advances made in the study of A_{2B} receptors and

[a] Address for correspondence: Italo Biaggioni, Division of Clinical Pharmacology, Vanderbilt University, Nashville, TN 37232-2195.

Dr. Feoktistov is the recipient of an American Lung Association Research Grant Award and an Asthma and Allergy Foundation of America Investigator Grant Award and is supported also by NIH grants RR00095 and R29HL55596.

Abbreviations: alloxazine, 2,4-dioxobenzo[g]pteridine; ATP, adenosine 5c-triphosphate; cAMP, adenosine 3c,5c-cyclic monophosphate; cDNA, complementary deoxyribonucleic acid; CGS 21680, 4-[(N-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; DPSPX, 1,3-dipropyl-8-(p-sulfophenyl)xanthine; EC_{50}, concentration that produces half-maximal effect; enprofylline, 3-n-propylxanthine; HEL, human erythroleukemia; IB-MECA, N^6-(3-iodobenzyl)-N-methyl-5′-carbamoyladenosine; IgE, immunoglobulin E; IL-8, interleukin-8; K_B, dissociation constant of antagonist-receptor complex; L-249313, 6-carboxymethyl-5,9-dihydro-9-methyl-2-phenyl-[1,2,4]-triazolo-{5,1-a}-[2,7]-naphthyridine; L-268605, 3-(4-methoxyphenyl)-5-amino-7-oxo-thiazolo-[3,2]-pyrimidine; L-NAME, N^G-nitro-L-arginine methyl ester; mRNA, messenger ribonucleic acid; MRS 1067, 3,6-dichloro-2′-isopropyloxy-4′-methylflavone; MRS 1097, 3,5-diethyl 2-methyl, 6-phenyl-4-[2-[phenyl-(trans)-vinyl]-1,4(±)dihydropyiridine-3,5-dicarboxylate; MRS 1191, 3-ethyl 5-benzyl 2-methyl-phenylethynyl-6-phenyl-1,4(±)dihydropyridine-3,5-dicarboxylate; MRS 1222, 3,5-diethyl 2-methyl-4-[2-(4-nitrophenyl)-(*E*)-vinyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate; NECA, 5′-N-ethylcarboxamidoadenosine; NO, nitric oxide; R-PIA, (R)-N^6-phenylisopropyladenosine; S-PIA, (S)-N^6-phenylisopropyladenosine; SCH 58261, 5-amino-7-(phenylethyl)-2-(1-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; WRC-0571, C^8-(N-methylisopropyl)-amino-N^6-(5′-endohydroxy)-endonorbornan-2-yl-9-methyladenin; XAC, xanthine amine congener; ZM 241385, 4-(2-[7-amino-2-)2-furyl(triazolo {2,3-a}-[1,3,5]triazin-5-ylamino]ethyl)phenol.

underscore areas in which more progress is needed. We discuss some of the characteristics of A_1, A_{2A}, and A_3 receptors only to highlight their similarities and differences with A_{2B} receptors. Recent reviews on specific adenosine receptor subtypes can be found elsewhere (Linden, 1991, 1994; Dalziel and Westfall, 1994; Fredholm, 1995; Palmer and Stiles, 1995; Sebastião and Ribeiro, 1996; Daval et al., 1996; Ongini and Fredholm, 1996).

II. Classification of Adenosine Receptors

The properties of extracellular adenosine as a protective autacoid have been known since the study of its cardiovascular effects conducted in 1929 by Drury and Szent-Györgyi (1929). The purinergic receptors that mediate the effects of adenosine were classified as P_1 receptors, whereas the receptors activated by nucleotides like adenosine 5c-triphosphate (ATP) were classified as P_2 receptors (Burnstock, 1978). Adenosine receptors were found to modulate intracellular levels of adenosine 3c,5c-cyclic monophosphate (cAMP) and were initially subdivided into A_1 and A_2 subtypes based on their ability to inhibit or stimulate adenyl cyclase, respectively (van Calker et al., 1979; Londos et al., 1980). The alternative classification of adenosine receptors as R_i and R_a (Londos et al., 1980) was replaced by the A_1 and A_2 terms (van Calker et al., 1979). The further division of A_2 receptors into two subtypes was proposed originally by Daly et al. (1983) based on the finding of high-affinity A_2 receptors in rat striatum and low-affinity A_2 receptors throughout the brain, both of which activated adenyl cyclase. The existence of subtypes of A_2 receptors was also suggested by the finding, independently reported by Elfman et al. (1984), of high-affinity A_2 receptors in cultured neuroblastoma cells and low-affinity A_2 receptors in glioma cells. These high- and low-affinity receptor subtypes were later designated as A_{2A} and A_{2B}, respectively (Bruns et al., 1986). The classification of P_1 receptors has been validated by the recent success in molecular cloning and expression of all three anticipated A_1, A_{2A}, and A_{2B} adenosine receptors and the previously unrecognized A_3 receptor (Maenhaut et al., 1990; Libert et al., 1991; Zhou et al., 1992; Rivkees and Reppert, 1992; Pierce et al., 1992). This classification has been endorsed by IUPHAR Committee on Receptor Nomenclature and Drug Classification (Fredholm et al., 1994, 1996b, 1997).

III. Molecular Characterization of A_{2B} Receptors

Adenosine A_{2B} receptors were cloned from rat hypothalamus (Rivkees and Reppert, 1992), human hippocampus (Pierce et al., 1992), and mouse mast cells (Marquardt et al., 1994), employing standard polymerase chain reaction techniques with degenerate oligonucleotide primers designed to recognize conserved regions of most G protein-coupled receptors. The human A_{2B} receptor shares 86 to 87% amino acid sequence homology with the rat and mouse A_{2B} receptors (Rivkees and Reppert, 1992; Pierce et al., 1992; Marquardt et al., 1994) and 45% amino acid sequence homology with human A_1 and A_{2A} receptors (fig. 1). As expected for closely related species, the rat and mouse A_{2B} receptors share 96% amino acid sequence homology. By comparison, the overall amino acid identity between A_1 receptors from various species is 87% (Palmer and Stiles, 1995). A_{2A} receptors share 90% of homology between species (Ongini and Fredholm, 1996), with most differences occurring in the 2nd extracellular loop and the long C-terminal domain (Palmer and Stiles, 1995). The lowest (72%) degree of identity between species is observed for A_3 receptor sequences (Palmer and Stiles, 1995). Differences in amino acid sequence of adenosine receptors between species may result in distinct pharmacological characteristics. For example, the rat A_1 receptor has a rank order of potency (R)-N^6-phenylisopropyladenosine (R-PIA) > 5′-N-ethylcarboxamidoadenosine (NECA) > (S)-N^6-phenylisopropylaolenesine (S-PIA), and the bovine A_1 receptor has a potency order R-PIA > S-PIA > NECA (Klotz et al., 1991), whereas the canine A_1 receptor binds NECA with a higher affinity than that of R-PIA (Tucker and Linden, 1993). The differences between amino acid sequences of A_3 receptors are reflected in the insensitivity of the rat A_3 receptor to antagonism by methylxanthines (Zhou et al., 1992), a phenomenon that is not observed in the human or sheep A_3 receptor (Linden et al., 1993; Salvatore et al., 1993). These interspecies differences between adenosine receptors explain why the adenosine agonist xanthine amine congener (XAC) is a selective A_1 agonist in the rat, but not in the human or rabbit (Jacobson et al., 1992; Jacobson and

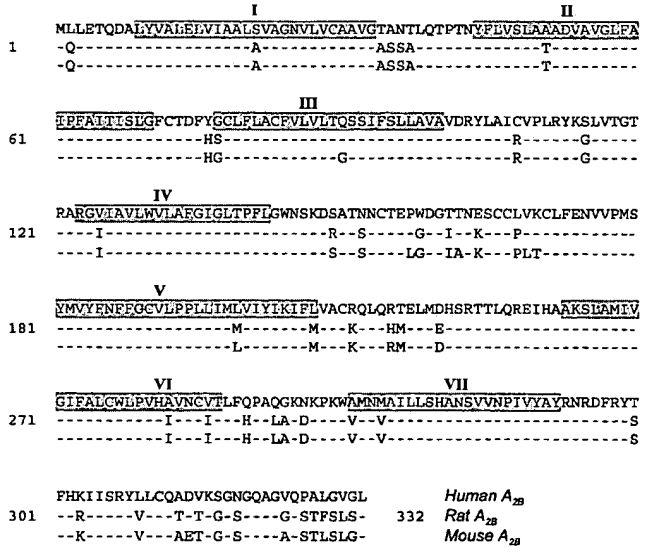


FIG. 1. Comparison of amino acid sequences of human (M97759) (Pierce et al., 1992), rat (M91466) (Stehle et al., 1992), and mouse (U05673) (Marquardt et al., 1994) A_{2B} receptors. Dashed lines indicate amino acid identity. Predicted transmembrane spanning domains are highlighted and indicated by roman numerals.

Suzuki, 1996). Few comparisons have been made between A_{2B} receptors from different species. No differences in pharmacological profiles were found between A_{2B} receptors from fibroblasts of murine and human origin (Bruns, 1981; Brackett and Daly, 1994) or between human A_{2B} receptor expressed in Chinese hamster ovary (CHO) cells and guinea pig brain A_{2B} receptors (Alexander et al., 1996).

The proposed membrane structure of A_{2B} receptors is typical of G protein–coupled receptors, with seven transmembrane domains connected by three extracellular and three intracellular loops, and flanked by an extracellular N-terminus and an intracellular C-terminus (Rivkees and Reppert, 1992; Pierce et al., 1992; Marquardt et al., 1994; fig. 2). The highest degree of identity in amino acid sequences between A_{2B} receptors of different species is found in the transmembrane domains (fig. 1). The 2nd extracellular loop of the human, mouse, and rat A_{2B} receptors contains two potential N-glycosylation sites (Rivkees and Reppert, 1992; Pierce et al., 1992; Marquardt et al., 1994). It should be noted that enzymatic treatment failed to demonstrate N-glycosylation of A_{2B} receptors in T84 epithelial cells (Puffinbarger et al., 1995). However, it is not clear whether A_{2B} receptors are glycosylated in other cells or glycosylation can alter A_{2B} function. A_{2A} receptors were found to be glycosylated in canine striatum and liver membranes (Palmer et al., 1992), but the binding characteristics of A_{2A} receptors for 4-[(N-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl]-phenylpropionic acid (CGS 21680) appear to be the same in both glycosylated or unglycosylated forms of the receptor expressed in COS M6 cells (Piersen et al., 1994).

The predicted molecular mass of A_{2B} receptors is similar to that of A_1 and A_3 receptors (36–37 kDa), whereas A_{2A} receptors have a larger predicted size (45 kDa). The greater molecular mass of the A_{2A} receptor is explained by the presence of a longer intracellular C-terminus. Together with the 3rd intracellular loop, the intracellular C-terminus is thought to be involved in the coupling of A_{2A} receptors to G proteins (Palmer and Stiles, 1995). To date, no mutational analysis of A_{2B} receptor-G protein coupling has been reported. However, some parallels could be drawn from studies using chimeric A_1/A_{2A} adenosine receptors (Tucker et al., 1996; Olah, 1997). Using this approach, it has been shown that the amino terminal portion of the 3rd intracellular loop of the A_{2A} receptor determines its selective coupling with G_s (Olah, 1997). This 15-mer portion of the A_{2A} receptor shares 57% amino acid sequence homology with the A_{2B} receptor, both of which are coupled to G_s, and only 27% with the A_1 receptor, which is not coupled to G_s (fig. 3). In addition, the nature of the amino acids in the 2nd intracellular loop may indirectly modulate A_{2A} receptor coupling. In particular, lysine and glutamic acid residues in that portion of the molecule were found to be necessary for efficient A_{2A} adenosine receptor-G_s coupling (Olah, 1997). These amino acid residues are also present in the A_{2B} receptor. The long intracellular C-terminal tail of the A_{2A} receptor, which represents a major structural difference with the A_{2B}, does not appear to be involved in the determination of receptor coupling to G_s protein. The removal of the C-terminal tail of the A_{2A} receptor, or its replacement with a cytoplasmic tail of the A_1 receptor, does not impair stimulation of adenyl cyclase when these truncated or chimeric receptors are expressed in CHO cells (Tucker et al., 1996; Palmer and Stiles, 1997; Olah, 1997). The data generated from these studies, however, leave the possibility that this region can still play a role in the modulation of the coupling of A_{2A} receptors to G proteins. For example, it was suggested that the C-terminal tail confers the A_{2A} receptors' ability to couple tightly to G_s, a feature considered to be unique for this receptor subtype (Nanoff et al., 1991).

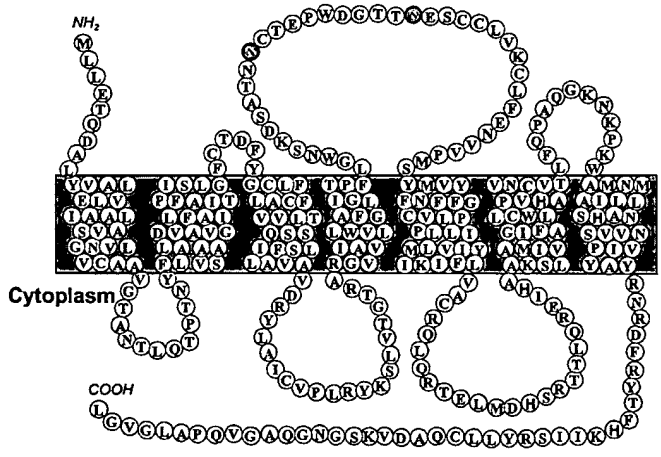


FIG. 2. Amino acid sequence of the human A_{2B} receptor. The receptor is drawn according to the seven-membrane spanning motif common to the G protein-coupled receptor superfamily. Possible sites of N-linked glycosylation on the 2nd extracellular loop are highlighted.

Second intracellular loop

104	---LRVK-----KMV--PR-	*Human A_1*
101	DRYIAIRIPLRYNGLVTGTR	*Human A_{2A}*
102	---L--CV----KS------	*Human A_{2B}*

Third intracellular loop

202	EV-YLI-K--NKK**VSASSGDPQKYYGKELKI--	*Human A_1*
199	RIFIAARRQLKQMESQPLPGERARSTLQKEVHAAK	*Human A_{2A}*
204	K---V-C---QRT-LM****DHS-T---R-I----	*Human A_{2B}*

Intracellular C-terminal tail

291	--QK--V--L--WND-FRC-PA-PIDEDLPEERPDD	*Human A_1*
291	RIREFRQTFRKIIRSHVLRQQEPFKAAGTSARVLAAHGSVGEQ	*Human A_{2A}*
293	-N-D--Y--H---SRYL-C-ADVKSGN-QAGVQP-LGVGL	*Human A_{2B}*
	VSLRLNGHPPEVWANGSAPHPERRPNGYALGLVSGGSAQESQG	*Human A_{2A}*
	NTGLPDVELLSHELKRVCPEPPGLDDPLAQDGAGVS	*Human A_{2A}*

FIG. 3. Comparison of amino acid sequences of 2nd and 3rd intracellular loops and C-terminus of human A_{2A} receptors, with corresponding regions of human A_1 and A_{2B} receptors. Dashed lines indicate amino acid identity. Asterisks represent gaps in the sequence introduced to highlight amino acid homologies. The amino acid residues discussed in the text are highlighted.

Mutational studies of A_{2A} receptors revealed that a threonine residue (Thr^{298}) of the C-terminal tail of the A_{2A} receptor, located in proximity to the seventh transmembrane span (fig. 3), is essential for the development of rapid agonist-mediated desensitization (Palmer and Stiles, 1997). This amino acid residue is also present in the human A_{2B} receptor (Thr^{300}), but its role in receptor desensitization has not been explored. Although the mechanisms of desensitization are not completely identified, it is of interest that rapid desensitization of A_{2A} as well as A_{2B} receptors can be mediated by G protein-coupled receptor kinase 2 (Mundell et al., 1997). It should be noted that A_{2B} receptors can be coupled to other intracellular signaling pathways in addition to G_s and adenyl cyclase. The similarities and differences in A_{2B} and A_{2A} receptor coupling to G proteins warrant studies involving mutational analysis of A_{2B} receptors, and possibly chimeric A_{2A}/A_{2B} receptors, to better understand determinants of A_{2B}-G protein coupling.

The human A_{2B} receptor gene was mapped to chromosome 17p11.2-p12 (Jacobson et al., 1995; Townsend-Nicholson et al., 1995). A single intron interrupts the coding sequence of the human A_{2B} receptor gene in a region corresponding to the 2^{nd} intracellular loop between $Leu^{111}$and Arg^{112} (Jacobson et al., 1995). In this respect, the human A_{2B} receptor gene is similar to the other human adenosine receptor genes in that it also contains a single intron in its coding sequence (Ren and Stiles, 1994; Peterfreund et al., 1994; Murrison et al., 1996). Some G protein-coupled receptors are known to have multiple introns in the coding sequences of their corresponding genes. Alternative splicing of their primary transcripts results in heterogeneity in protein sequences, as observed with EP_3 prostanoid receptors (Neglishi et al., 1995), D_2 dopamine receptors (Giros et al., 1989), lutropin/choriogonadotropin receptors (Aatsinki et al., 1992), and fibroblast growth factor receptors 2 (Dell and Williams, 1992). The presence of only one intron within the coding region of the human A_{2B} receptor gene precludes structural variations of A_{2B} receptors by alternative splicing.

In addition to the human A_{2B} receptor gene, an A_{2B} pseudogene with 79% identity with the A_{2B} receptor complementary deoxyribonucleic acid (cDNA), has been localized to chromosome 1q32 (Jacobson et al., 1995; Townsend-Nicholson et al., 1995). When compared with the coding sequence of the A_{2B} receptor, the pseudogene contained multiple deletions, point mutations, and frame shifts and two in-frame stops (Jacobson et al., 1995). It is doubtful that with all these changes the pseudogene would encode a functional adenosine receptor. However, further studies are needed to determine whether the A_{2B} pseudogene is transcriptionally competent. For example, dopamine D_5 pseudogene transcripts can be detected in human brain tissues (Nguyen et al., 1991). The same possibility should always be considered in Northern blot analysis or in situ hybridization of A_{2B} receptor in various tissues, because the use of sequences common between the functional A_{2B} cDNA and the A_{2B} pseudogene as probes could potentially lead to misinterpretation of results.

IV. Pharmacology of A_{2B} Receptors

Highly selective and potent agonists have been designed for A_1, A_{2A}, and A_3 receptors. These compounds have been important tools in the characterization of adenosine receptors and the determination of their functions. All four subtypes, including A_{2B} receptors, have a typical order of potency for agonists (table 1; fig. 4). However, no selective agonist for A_{2B} receptors has been found so far. The adenosine analog NECA remains the most potent A_{2B} agonist (Bruns, 1981; Feoktistov and Biaggioni, 1993, 1997; Brackett and Daly, 1994), with a concentration producing a half-maximal effect (EC_{50}) for stimulation of adenyl cyclase of approximately 2 μM. It is, however, nonselective and activates other adenosine receptors with even greater affinity, with an EC_{50} in the low nanomolar (A_1 and A_{2A}) or high nanomolar (A_3) range (table 1; fig. 4). The characterization of A_{2B} receptors, therefore, often relies on the lack of effectiveness of compounds that are potent and selective agonists of other receptor types. A_{2B} receptors have been characterized by a method of exclusion, i.e., by the lack of efficacy of agonists that are specific for other receptors. The A_{2A} selective agonist CGS 21680 (Webb et al., 1992), for example, has been useful in differentiating between A_{2A} and A_{2B} adenosine receptors (Hide et al., 1992; Chern et al., 1993; Feoktistov and Biaggioni, 1995; van der Ploeg et al., 1996). Both receptors are positively coupled to adenyl cyclase and are activated by the nonselective agonist NECA. CGS 21680 is virtually ineffective on A_{2B} receptors but is as potent as NECA in activating A_{2A} receptors, with an EC_{50} in the low nanomolar range for both agonists (Jarvis et al., 1989; Nakane and Chiba, 1990; Webb et al., 1992; Hide et al., 1992; Feoktistov and Biaggioni, 1993; Alexander et al., 1996). A_{2B} receptors have also a very low affinity for the A_1 selective agonist R-PIA (Feoktistov and Biaggioni, 1993; Brackett and Daly, 1994) as well as for the A_3 selective agonist N^6-(3-iodobenzyl)-N-methyl-5′-carbamoyladenosine (IB-MECA) (Feoktistov and Biaggioni, 1997). The agonist profile NECA > R-PIA = IB-MECA > CGS 21680 was determined in human erythroleukemia (HEL) cells for A_{2B}-mediated cAMP accumulation. The difference between EC_{50} for NECA and the rest of the agonists is approximately 2 orders of magnitude. Therefore, responses elicited by NECA at concentrations in the low micromolar range (1–10 μM), but not by R-PIA, IB-MECA or CGS 21680, are characteristic of A_{2B} receptors.

Pharmacological characterization of receptors based on apparent agonist potencies, however, is far from ideal, because it depends not only on agonist binding to the receptor but also on multiple processes involved in

TABLE 1
Pharmacological characteristics of adenosine receptor subtypes

Adenosine receptor	Order of potency for agonists[a] (μM)	Selective agonists[b]	Selective antagonists[b]
A_1	R-PIA (0.001) > NECA (0.006) > IB-MECA (0.054) > CGS 21680 (2.6)	R-PIA, CPA	DPCPX, N-0861
A_{2A}	NECA (0.01) = CGS 21680 (0.015) > IB-MECA (0.056) > R-PIA (0.124)	CGS 21680, APEC	SCH 58261, ZM 241385
A_{2B}	NECA (2) > R-PIA (160) = IB-MECA (201) > CGS 21680 (1600)	None	Enprofylline
A_3	IB-MECA (0.001) > NECA (0.113) = R-PIA (0.158) > CGS 21680 (0.584)	IB-MECA, Cl-IB-MECA	MRS 1067, MRS 1097 L-249313, L-268605

[a] Data shown for rat A_1, A_{2A}, and A_3 receptors are K_i values based on radioligand binding, from van Galen et al. (1994) and Gallo-Rodriguez et al. (1994). Data shown for A_{2B} receptors are EC_{50} values for cAMP accumulation in human erythroleukemia cells from Feoktistov and Biaggioni (1993) and Feoktistov and Biaggioni (1997a).
[b] Data are derived from Feoktistov and Biaggioni (1995), Palmer and Stiles (1995), Jacobson et al. (1996), and Jacobson (1996).

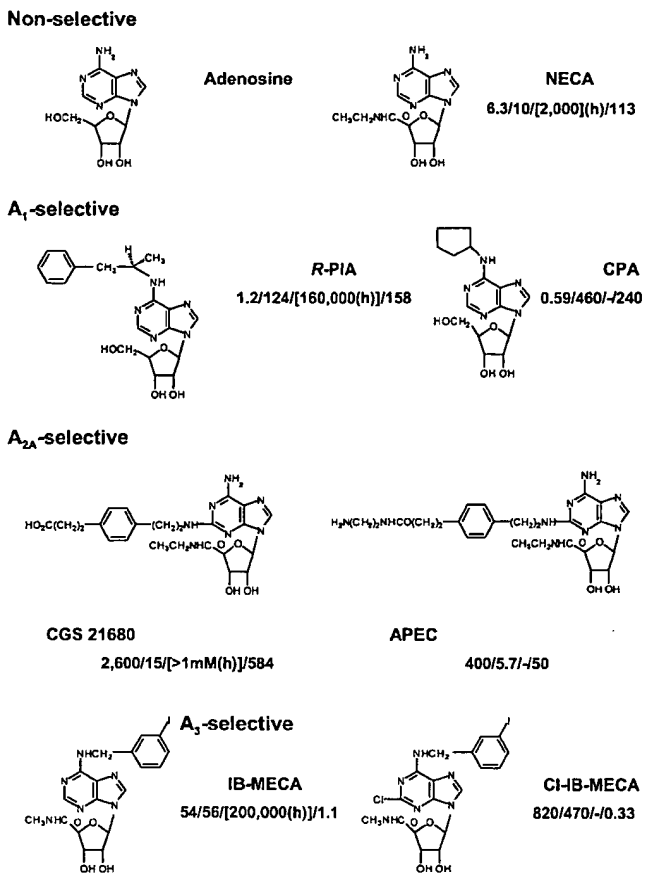


FIG. 4. Chemical structure and radioligand binding data on the affinity of adenosine agonists. K_i values (nM) for rat $A_1/A_{2A}/A_{2B}/A_3$ receptors are shown, except as indicated (h, human). Numbers in brackets represent EC_{50} of adenosine agonists for cAMP accumulation in HEL cells. Data compiled from Feoktistov and Biaggioni (1993), van Galen et al. (1994), Jacobson and Suzuki (1996), and Feoktistov and Biaggioni (1997).

signal transduction. Selective antagonists are preferable for receptor subtype identification (Kenakin et al., 1992). Highly selective and potent A_{2B} antagonists are not yet available, but, whereas A_{2B} receptors have a lower affinity for agonists compared with other receptor subtypes, this is not true for antagonists. The structure-activity relationship of A_{2B} receptors for adenosine antagonists has not been completely characterized, but at least some xanthines are as potent antagonists at A_{2B} receptors as at other adenosine receptors (Feoktistov and Biaggioni, 1993; Brackett and Daly, 1994).

The antiasthmatic drug enprofylline (3-n-propylxanthine), is the most selective A_{2B} antagonist known to date. In early studies, enprofylline was found to be about 20 times more potent in blocking hippocampal A_2 receptors compared with rat fat cell A_1 receptors (Fredholm and Persson, 1982). It was initially proposed, therefore, that enprofylline can selectively block a subtype of A_2 receptors in the hippocampus (Fredholm and Persson, 1982). However, enprofylline was then found to be a poor antagonist of A_2 receptors in thymocytes (Fredholm and

Sandberg, 1983) and platelets (Ukena et al., 1985). More recently, enprofylline has also been found to have a low affinity for A_3 receptors (Linden et al., 1993). These findings led to the conclusion that enprofylline was not an adenosine receptor antagonist. These original studies need to be reinterpreted in the light of our current knowledge of adenosine receptor subtypes. It is now known that accumulation of cAMP in hippocampal slices, which was shown to be blocked by enprofylline, is mediated by A_{2B} receptors (Lupica et al., 1990), and that platelets, found to be insensitive to enprofylline, express mainly A_{2A} receptors (Feoktistov and Biaggioni, 1993; Dionisotti et al., 1996; Ledent et al., 1997). Therefore, previous contradictory results can now be explained by a selective antagonism of A_{2B} receptors by enprofylline. Indeed, it was recently demonstrated that enprofylline is equipotent to theophylline as an A_{2B} receptor antagonist in HEL cells, with a dissociation constant of antagonist-receptor complex (K_B) of 7 μM (Feoktistov and Biaggioni, 1995). An analysis of the original results in the hippocampus (Fredholm and Persson, 1982) reveals an approximate K_B of 6 μM. An identical K_i for enprofylline (7 μM) was found in CHO cells stably transfected with A_{2B} using radioligand binding with [^{3}H]1,3 diethyl-8-phenylxanthine (Robeva et al., 1996). This value also correlated well with the K_B estimated from inhibition of NECA-induced cAMP generation in a similar cell model (23 μM) (Alexander et al., 1996). Enprofylline is also an effective antagonist of A_{2B} receptors in human HMC-1 mast cells (Feoktistov and Biaggioni, 1995) and canine BR mastocytoma cells (Auchampach et al., 1996). In comparative radioligand binding studies on all four human adenosine receptors permanently expressed in CHO cells, enprofylline has been shown to be 22-fold selective for A_{2B} versus A_1, five-fold versus A_{2A}, and six-fold versus A_3 (Robeva et al., 1996). Enprofylline, therefore, can be considered a relatively selective, though not potent A_{2B} antagonist.

More potent but nonselective A_{2B} receptor antagonists have been also characterized. These compounds include 1,3-dipropyl-8-(p-sulfophenyl)xanthine (DPSPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), and XAC (Feoktistov and Biaggioni, 1993; Brackett and Daly, 1994). The xanthine antagonist DPSPX is 20-fold more potent at A_{2B} receptors in HEL cells (K_B = 141 nM) compared with platelet A_{2A} receptors (Feoktistov and Biaggioni, 1993). However, the affinity of A_{2B} receptors for DPSPX (Feoktistov and Biaggioni, 1993) is similar to those of sheep A_3 (Linden et al., 1993) and rat A_1 (Ukena et al., 1986) receptors. Among nonxanthine compounds, 2,4-dioxobenzo[g]pteridine (alloxazine) was reported to be nine-fold more potent as an antagonist of A_{2B} receptors in VA13 and NIH 3T3 cells compared with A_{2A} receptors in PC12 cells (Brackett and Daly, 1994; fig. 5).

A_{2B} receptors are frequently found with other adenosine receptor subtypes in the same tissue, and are even coexpressed in the same cells. Recent advances in the development of selective A_1, A_{2A}, and A_3 antagonists (table 1; fig. 5) provide a new approach to the study of A_{2B} receptors; the nonselective agonist NECA can be used in conjunction with highly selective antagonists of other adenosine receptor subtypes to selectively stimulate A_{2B} receptors. The ability to selectively block other adenosine receptors is particularly useful in situations in which they are present with A_{2B} receptors.

The first selective A_1 antagonist DPCPX was discovered by two independent groups of investigators (Martinson et al., 1987; Bruns et al., 1987) and has become the reference A_1 receptor antagonist. It is highly selective for A_1 versus A_{2A} (80- to 500- fold across species) (Jacobson et al., 1992; Robeva et al., 1996). In recent radioligand binding studies involving all four human recombinant adenosine receptors, DPCPX has been confirmed to be 20-fold selective for A_1 versus A_{2B} (Robeva et al., 1996). Selective blockade of A_1 receptors with DPCPX was successfully used to reveal functional A_{2B} receptors in tissues coexpressing both A_1 and A_{2B} receptors (Mogul et al., 1993; Murthy et al., 1995; Nicholls et al., 1996). Other compounds have been identified with even greater selectivity for the A_1 receptor; C^8-(N-methylisopropyl)-amino-N^6-(5′-endohydroxy)-endonorbornan-2-yl-9-methyladenin (WRC-0571) binds to human A_1 receptors with a K_i of 3 nM and to human A_{2B} receptors with a K_i of 19 μM. This compound, therefore, is approximately 6300-fold selective for A_1 versus A_{2B} (Robeva et al., 1996).

Among the new generation of A_{2A} antagonists, 4-(2-[7-amino-2-)2-furyl(triazolo {2,3-a}-[1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) was reported to be 30- to 80-fold selective for A_{2A} versus A_{2B} (Poucher et al., 1995). Another antagonist, 5-amino-7-(phenylethyl)-2-(1-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261), has a high affinity (K_i = 0.7–2.2 nM) for A_{2A} receptors (Belardinelli et al., 1996; Lindström et al., 1996; Dionisotti et al., 1996; Zocchi et al., 1996a,b; Ongini et al., 1996; Ongini and Fredholm, 1996) but was found not to block NECA-induced vasorelaxation of guinea pig aorta, a process thought to be mediated by A_{2B} receptors (Zocchi et al., 1996a). The selectivity of SCH 58261 for A_{2A} versus A_{2B} has been also confirmed in a cellular system; this compound was ineffective on HEL cell A_{2B} receptors up to a concentration of 100 nM, whereas it inhibited the CGS 21680-induced cAMP accumulation in HMC-1 cell (A_{2A} receptor) with a K_B of 0.1 nM (Feoktistov and Biaggioni, 1997). SCH 58261, therefore, can be useful in the discrimination of A_{2B} function in cells also coexpressing A_{2A} receptors. This approach was applied to the study of adenosine receptors in the human mast cells HMC-1 (fig. 6). The concentration-response relationship of the nonselective adenosine agonist NECA for cAMP accumulation in these cells follows a curve with a Hill slope of 0.64 ± 0.07 best fitted to a two-site model with an apparent pD_2 of 7.69 ± 0.42 and 5.92 ± 0.21 for the high- and low-affinity sites,

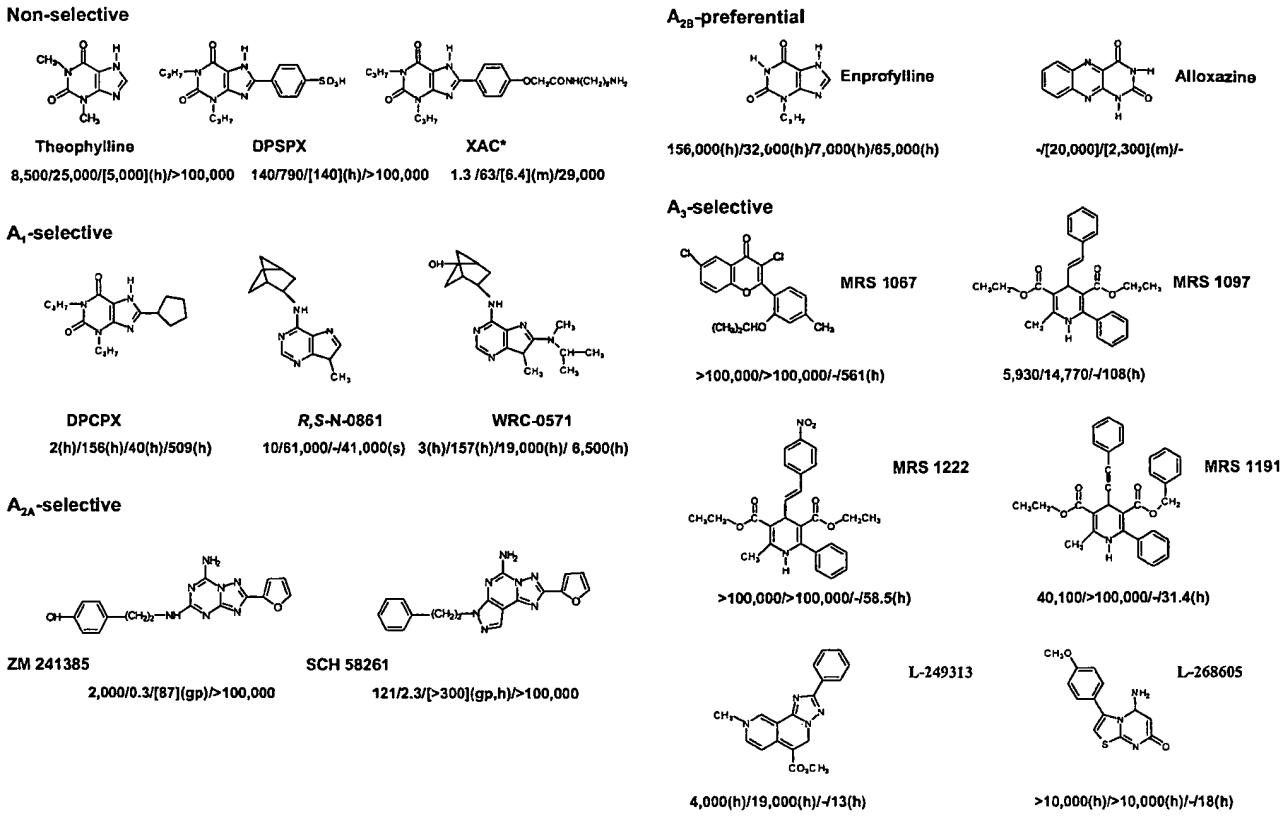


FIG. 5. Chemical structure and radioligand binding data on the affinity of adenosine antagonists. K_i values (nM) for rat $A_1/A_{2A}/A_{2B}/A_3$ receptors are shown, except as indicated (h, human; gp, guinea pig; s, sheep; m, mice). Numbers in brackets represent K_B of adenosine antagonists. Data compiled from Feoktistov and Biaggioni (1993), Brackett and Daly (1994), van Galen et al. (1994), Jacobson et al. (1996), Robeva et al. (1996), Jiang et al. (1996), van Rhee et al. (1996), Zocchi et al. (1996a), and Jacobson and Suzuki (1996). *, selective for rat, but not for human or rabbit A_1 receptors (Jacobson and Suzuki, 1996).

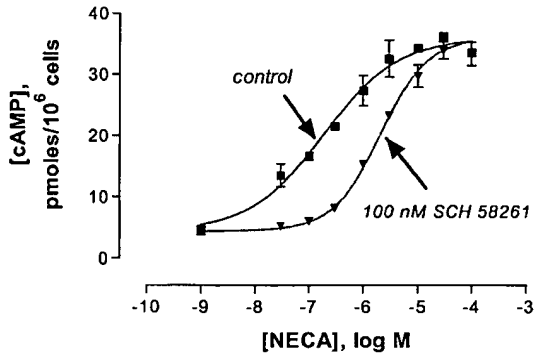


FIG. 6. Selective activation of A_{2B} receptors with NECA in HMC-1 human mast cells coexpressing A_{2A} and A_{2B} receptors, made possible by blockade of A_{2A} receptors with the selective antagonist SCH 58261 (Feoktistov and Biaggioni, 1997). See text for details.

respectively. Upon complete blockade of A_{2A} receptors with 100 nM SCH 58261, the concentration-response curve of NECA was transformed into a typical sigmoid curve with a Hill slope of 0.93 ± 0.06 and a pD_2 of 5.68 ± 0.03, consistent with activation of A_{2B} receptors. Blockade of A_{2A} receptors in the same cells with SCH 58261 did not affect NECA-induced calcium mobilization, confirming that this process is mediated solely via A_{2B} receptors (Feoktistov and Biaggioni, 1997), as it has been previously suggested on the basis of the lack of CGS 21680 effectiveness (Feoktistov and Biaggioni, 1995).

Recently, several antagonists with A_3 selectivity versus A_1 and A_{2A} receptors have been introduced. These compounds include the flavonoid derivative 3,6-dichloro-2′-isopropyloxy-4′-methylflavone (MRS 1067) and the dihydropyridine derivatives 3-ethyl 5-benzyl 2-methyl-phenylethynyl-6-phenyl-1,4(±)dihydropyridine-3,5-dicarboxylate (MRS 1191), 3,5-diethyl 2-methyl-4-[2-(4-nitrophenyl)-(*E*)-vinyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1222), and 3,5-diethyl 2-methyl, 6-phenyl-4-[2-[phenyl-(trans)-vinyl]-1,4(±)dihydropyiridine-3,5-dicarboxylate (MRS 1097) (fig. 5), which are selective for the human A_3 receptor by a factor of 45- to 1700-fold, versus rat A_1 and A_{2A} receptors, as determined from radioligand binding studies (Jiang et al., 1996; Karton et al., 1996; van Rhee et al., 1996). It should be noted, however, that the highest degree of selectivity for these compounds is observed when their effects on human A_3 receptors are compared with their effects on rat A_1 and A_{2A} receptors. For example, MRS

1191 was selective for the human A_3 receptor by factor of 1300-fold, whereas for the rat A_3 receptor, the selectivity was only 11-fold versus the rat A_1 receptor (Jiang et al., 1996). Among other compounds, the triazolonaphthyridine derivative 6-carboxymethyl-5,9-dihydro-9-methyl-2-phenyl-[1,2,4]-triazolo-{5,1-a}-[2,7]-naphthyridine (L-249313) (fig. 5) is highly potent on human A_3 receptors (K_i =13 nM), but not on rat A_3 receptors (K_i = 58 μM). The thiazolopyrimidine derivative 3-(4-methoxyphenyl)-5-amino-7-oxo-thiazolo-[3,2]-pyrimidine (L-268605) was also shown to be a potent antagonist on human A_3 receptor (K_i = 18 nM). Both compounds are highly selective for the human A_3 receptor versus the human A_1 (>300-fold) and A_{2A} (>1400-fold) receptors (Jacobson et al., 1996). Unfortunately, A_{2B} receptors have not been included when characterizing the selectivity of A_3 antagonists. Additional studies of A_3 antagonists with respect to A_{2B} receptors are required to verify whether they can be useful to discriminate between A_3 and A_{2B}-mediated effects.

In summary, potent and selective agonists and antagonists are available for all adenosine receptors except for the A_{2B} subtype. The characterization of A_{2B} receptors has been based on apparent potencies of agonists selective to other adenosine receptor subtypes. The development of selective A_1, A_{2A}, and A_3 antagonists provides a new approach when used in conjunction with the nonselective agonist NECA to selectively stimulate A_{2B} receptors. This approach is particularly useful in tissues or cells expressing more than one adenosine receptor. However, much progress in this field could be achieved by the development of selective A_{2B} receptor antagonists. Because of the low affinity of this receptor for agonists, the design of selective and potent A_{2B} antagonists seems to be more promising than the development of selective agonists.

V. Distribution of A_{2B} Receptors

The generation of cDNA for A_{2B} receptors has made possible the identification of the tissue distribution of this receptor subtype. A_{2B} receptor messenger ribonucleic acid (mRNA) was originally detected in a limited number of rat tissues by Northern blot analysis, with the highest levels found in cecum, bowel, and bladder, followed by brain, spinal cord, lung, epididymis, vas deferens, and pituitary (Stehle et al., 1992). The use of more sensitive reverse transcriptase-polymerase chain reaction techniques revealed a ubiquitous distribution of A_{2B} receptors. mRNA encoding A_{2B} receptors was detected at various levels in all rat tissues studied, with the highest levels in the proximal colon and lowest in the liver (Dixon et al., 1996). In situ hybridization of A_{2B} receptors showed widespread and uniform distribution of A_{2B} mRNA throughout the brain (Stehle et al., 1992; Dixon et al., 1996). The expression of A_{2B} receptors in a variety of human and murine tissues has been confirmed by Western blotting and by immunostaining with an anti-A_{2B} receptor antibody (Puffinbarger et al., 1995).

Pharmacological identification of A_{2B} receptors, based on their low affinity and characteristic order of potency for agonists, also indicates a widespread distribution of A_{2B} receptors. In brain, functional A_{2B} receptors are found in neurons (Mogul et al., 1993; Okada et al., 1996; Kessey et al., 1997) and glial cells (van Calker et al., 1979; Elfman et al., 1984; Hösli and Hösli, 1988; Altiok et al., 1992; Peakman and Hill, 1994, 1996; Fiebich et al., 1996a). Although there is no evidence that A_{2B} receptor are present in microglia (Fiebich et al., 1996b), there is ample data that show that they are expressed in astrocytes and in different glioma cell lines (Elfman et al., 1984; Hösli and Hösli, 1988; Altiok et al., 1992; Peakman and Hill, 1994, 1996; Fiebich et al., 1996a). The expression of A_{2B} receptors in glial cells, which represent a majority of the brain cell population, can explain the original observation that slices from all brain areas examined showed an adenosine-stimulated cAMP response (Sattin and Rall, 1970; Daly, 1976).

Functional A_{2B} receptors have been found in fibroblasts (Brackett and Daly, 1994) and various vascular beds (Martin, 1992; Martin et al., 1993; Chiang et al., 1994; Martin and Potts, 1994; Haynes et al., 1995; Rubino et al., 1995; Prentice and Hourani, 1996; Dubey et al., 1996b). Contamination with these cells may also contribute to the widespread pattern of A_{2B} receptor distribution in all organs. This possibility should always be considered, especially when data from crude tissue preparations are analyzed. The presence of functional A_{2B} receptors also has been demonstrated in hematopoietic cells (Feoktistov and Biaggioni, 1993; Porzig et al., 1995), mast cells (Marquardt et al., 1994; Feoktistov and Biaggioni, 1995), myocardial cells (Liang and Haltiwanger, 1995), intestinal epithelial (Strohmeier et al., 1995) and muscle cells (Murthy et al., 1995; Nicholls et al., 1996), retinal pigment epithelium (Blazynski, 1993; Gregory et al., 1994), endothelium (Iwamoto et al., 1994), and neurosecretory cells (Casado et al., 1992; Gharib et al., 1992; Mateo et al., 1995).

Coexpression of A_{2B} receptors together with other adenosine receptors has been reported in various cell preparations and cell lines. Functionally coupled A_{2B} and A_{2A} receptors are coexpressed in rat pheochromocytoma PC12 cells (Hide et al., 1992; Chern et al., 1993; van der Ploeg et al., 1996), T-cell leukemia Jurkat cells (van der Ploeg et al., 1996), mouse bone marrow-derived mast cells (Marquardt et al., 1994), human mast HMC-1 cells (Feoktistov and Biaggioni, 1995), human aortic endothelial cells (Iwamoto et al., 1994), human umbilical vein endothelial cells (Feoktistov and Biaggioni, unpublished observations), and human neutrophil leukocytes (Fredholm et al., 1996c). mRNA encoding A_{2A}, A_{2B}, and A_3, but not A_1 receptors, have been found in rat RBL 2H3 mast cells (Ramkumar et al., 1993; Marquardt et al., 1994).

Functional A_1 receptors can also be coexpressed with A_{2A} and/or A_{2B} receptors. In most cases, a selective blockade of A_1 receptors is required to unmask functional A_{2B} receptors. This approach was successfully used in dispersed guinea pig small intestinal muscle cells (Murthy et al., 1995), in rat duodenum longitudinal muscle muscularis mucosae cells (Nicholls et al., 1996), and in guinea pig pyramidal neurons from the hippocampal CA3 region (Mogul et al., 1993). Similarly, uncoupling of A_1 receptor using pertussis toxin unmasks the presence of A_{2A} and A_{2B} receptors in ventricular myocytes (Liang and Haltiwanger, 1995). By contrast, it was not necessary to block A_1 receptors in various glial cells to observe either A_{2A} or A_{2B} receptor-mediated stimulation of adenyl cyclase (Elfman et al., 1984; Altiok et al., 1992; Peakman and Hill, 1994, 1996; Fiebich et al., 1996a). Also, the balance between A_1- and A_2-mediated responses can be modulated. For example, corticosteroid treatment of DDT_1 MF2 smooth muscle cells increased A_1 receptor number and signaling and decreased A_2 receptor signaling (Gerwins and Fredholm, 1991). A similar decrease in the A_{2B} signaling upon dexamethasone treatment was also reported in Jurkat cells (Svenningsson and Fredholm, 1997).

Coexistence of different adenosine receptor types in cells obtained from primary tissue cultures (Iwamoto et al., 1994; Peakman and Hill, 1994, 1996) may be attributed to the presence of different subpopulations of cells, each one expressing a single type of adenosine receptor. However, studies on established cell lines (Hide et al., 1992; Feoktistov and Biaggioni, 1995; van der Ploeg et al., 1996) have confirmed the coexpression of adenosine receptors in a single target cell. Moreover, studies performed on single cells have also demonstrated the presence of more than one adenosine receptor subtype (Liang and Morley, 1996; Strickler et al., 1996), including A_{2B} receptors (Liang and Haltiwanger, 1995).

Coexpression of A_{2B} and A_{2A} receptors has been demonstrated even in clonal cell lines originally used to describe prototypic A_{2A} (PC12 cells) and A_{2B} receptors (Jurkat cells) (van der Ploeg et al., 1996). These cells predominantly express A_{2A} and A_{2B} receptors, respectively, and the presence of the other receptor type was recognized only after carefully conducted studies using differential responses to a series of 2-substituted adenosine analogs (Hide et al., 1992; van der Ploeg et al., 1996). It is entirely possible, therefore, that more examples of cells coexpressing adenosine receptors may become apparent after selective adenosine antagonists are applied in the characterization of these cells.

The functional meaning of this simultaneous expression of multiple adenosine receptor subtypes in a single target cell is not known. Because A_1 and A_{2A} receptors have a higher affinity for adenosine, in many cellular systems, these receptors need to be blocked before A_{2B}-mediated effects are apparent (Mogul et al., 1993; Liang and Haltiwanger, 1995; Murthy et al., 1995; Nicholls et al., 1996; Kessey et al., 1997; Feokstistov and Biaggioni, 1997). This, however, is not always the case. Both A_1 and A_{2B} receptors are present in glial cells of rat astrocytes, and stimulation of A_{2B} receptors with the nonselective agonist NECA induces cAMP accumulation that is evident even in the presence of A_1 receptors (Elfman et al., 1984; Altiok et al., 1992; Peakman and Hill, 1994, 1996; Fiebich et al., 1996a). It is possible that the relative importance of A_{2B} receptors is greater in situations in which high interstitial levels of adenosine are reached, e.g., in tissues in which metabolic demands are increased or oxygen supply is decreased, whereas the high affinity A_1 and A_{2A} receptors may modulate cellular functions in response to lower concentrations of this autacoid. The recent recognition that in cells coexpressing other adenosine receptors, A_{2B} receptors can be coupled to distinct intracellular pathways (Feoktistov and Biaggioni, 1995), may also provide the basis for a differential physiological role.

VI. Intracellular Pathways Regulated by A_{2B} Receptors

It is generally accepted that A_{2A} and A_{2B} receptors are coupled to G_s proteins, because both activate adenyl cyclase in virtually every cell in which they are expressed. Although activation of adenyl cyclase is arguably an important signaling mechanism for A_{2A} receptors, this is not necessarily the case for A_{2B} receptors, as other intracellular signaling pathways have been found to be functionally coupled to A_{2B} receptors in addition to adenyl cyclase (fig. 7).

Recombinant rat A_{2B} receptors expressed in *Xenopus* oocytes activate calcium-dependent chloride conductance presumably by stimulation of phospholipase C (Yakel et al., 1993). Likewise, it has been proposed that A_{2B} receptors stimulate phospholipase C in mouse bone marrow-derived mast cells (Marquardt et al., 1994). Regulatory proteins of the G_q family are thought to play a role in the coupling of A_{2B} receptors to β-phospholipase C in human mast HMC-1 cells (Feoktistov and Biaggioni, 1995) and canine BR mastocytoma cells (Auchampach et al., 1996), because this process is unaffected by treatment with pertussis or cholera toxins. A_{2B} receptor-mediated stimulation of β-phospholipase C results in mobilization of intracellular calcium in HMC-1 cells and eventually promotes synthesis of interleukin-8 (IL-8) (Feoktistov and Biaggioni, 1995; fig. 7a). In contrast to A_{2B} receptors, there is no evidence that A_{2A} receptors can stimulate phospholipase C under physiological conditions, even though cotransfection of human A_{2A} receptors with murine $G\alpha_{15}$ and human $G\alpha_{16}$, but not with $G\alpha_q$, $G\alpha_{11}$ or $G\alpha_{14}$, results in A_{2A}-mediated stimulation of phospholipase C in COS-7 cells (Offermanns and Simon, 1995). However, promiscuous coupling of $G\alpha_{15}$ and $G\alpha_{16}$ has been observed when these G proteins are coexpressed with receptors which are otherwise not normally coupled to phospholipase C (Milligan et al., 1996).

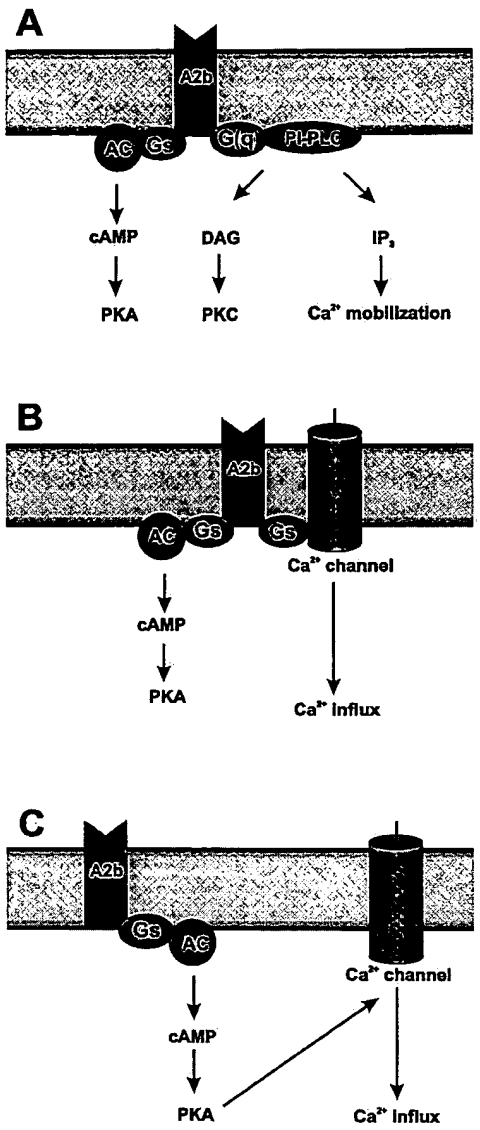


FIG. 7. Schematic representation of intracellular pathways coupled to adenosine A_{2B} receptors in various cells. A_{2B} receptors are coupled to adenyl cyclase (AC) in all cells shown. Activation of this pathway results in accumulation of cAMP and stimulation of protein kinase A (PKA). (A) A_{2B} receptors are coupled to phosphatidylinositol-specific phospholipase C (PI-PLC) via a G protein of the G_q family [$G_{(q)}$] in mast cells (Marquardt et al., 1994; Feoktistov and Biaggioni, 1995; Auchampach et al., 1996). Activation of this pathway results in increase in diacylglycerol (DAG) and inositol trisphosphate (IP_3). Diacylglycerol stimulates protein kinase C (PKC). Inositol trisphosphate activates mobilization of calcium from intracellular stores. (B) A_{2B} receptors potentiate calcium influx directly by coupling with G_s protein in HEL cells (Feoktistov et al., 1994). (C) In contrast, A_{2B} receptors potentiate calcium influx via cAMP and activation of protein kinase A in pyramidal neurons from the CA3 region of guinea pig hippocampus (Mogul et al., 1993).

Also, expression of $G\alpha_{15}$ and $G\alpha_{16}$ is limited only to a subset of hematopoietic cells (Amatruda et al., 1991; Wilkie et al., 1991).

Stimulation of A_{2B} receptors also increases intracellular calcium in HEL cells but not through a mechanism involving phospholipase C activation (fig. 7b). In contrast to the cholera toxin- and pertussis toxin-insensitive mobilization of intracellular calcium observed in HMC-1 mast cells, A_{2B} receptors facilitate calcium influx through a cholera toxin-sensitive mechanism in HEL cells. This effect was observed only when intracellular calcium levels were elevated, either by receptor-dependent (e.g., by thrombin) or -independent (e.g., thapsigargin) mechanisms. Even though this process is coupled to G_s-proteins, it is cAMP-independent. It has been suggested that αG_s, coupled to A_{2B} receptors, can directly stimulate a putative calcium channel (Feoktistov et al., 1994), as proposed for other G_s-coupled receptors (Imoto et al., 1988; Scamps et al., 1992).

Of interest, a similar mechanism has been suggested for A_{2A} receptors in fetal chicken ventricular myocardium cells. These cells coexpress A_{2A} and A_{2B} receptors, and both are positively coupled to stimulation of adenyl cyclase and myocyte contractility (Liang and Haltiwanger, 1995). Selective activation of A_{2A} receptors with CGS 21680 results in cAMP-independent calcium entry in pertussis toxin-treated cells. This effect does not involve simulation of phospholipase C and was blocked by the selective A_{2A} antagonist 8-(3-chlorostyryl)caffeine (Liang and Morley, 1996). This study did not explore the possibility that A_{2B} receptors share a common mechanism of G_s-mediated stimulation of calcium entry with A_{2A} receptors. This could be tested by using the nonspecific A_2 agonist NECA in the presence of a selective A_{2A} antagonist such as SCH 58261.

In another example of positive modulation of intracellular calcium, it has been reported that activation of A_{2B} receptors results in significant potentiation of P-type, but not N-type, calcium currents in pyramidal neurons from the CA3 region of guinea pig hippocampus. This mechanism was thought to be mediated by adenyl cyclase, because this potentiation could be inhibited by blocking the cAMP-dependent protein kinase (Mogul et al., 1993; fig. 7c).

It has recently been recognized that intracellular signaling of A_{2B} receptors can be modulated by interaction with other receptor systems (Fredholm, 1995; Fredholm et al., 1996a; fig. 8). For example, agents that increase intracellular calcium or activate protein kinase C significantly potentiate A_{2B}-mediated cAMP production in various cells (Hollingsworth et al., 1985; Norstedt and Fredholm, 1987; Fredholm et al., 1987; Norstedt et al., 1989; Kvanta et al., 1989, 1990; Altiok et al., 1992; fig. 8a). On the other hand, bradykinin-stimulated calcium entry caused inhibition of A_{2B} receptor-stimulated adenyl cyclase in astrocytoma D384 cells (fig. 8b), but direct stimulation of protein kinase C enhanced the A_{2B} response (Altiok et al., 1992; Altiok and Fredholm, 1993). The exact mechanism of the interaction between protein kinase C and A_{2B}-mediated pathways is not known, but it cannot be considered a unique feature of A_{2B} receptors. For instance, activation of thrombin-induced phos-

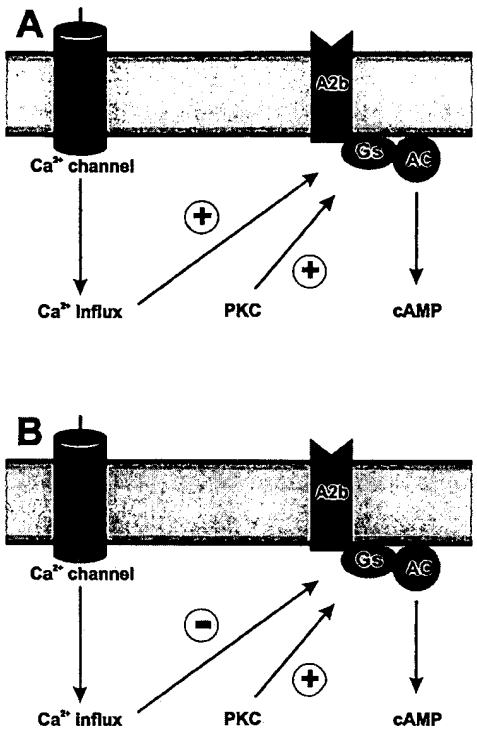


FIG. 8. Modulation of A_{2B} receptor signaling. (A) In Jurkat cells, stimulation of calcium entry and of protein kinase C (PKC) by T-cell receptor activation increases the magnitude of the cAMP response to stimulation of A_{2B} receptors (Kvanta et al., 1989; Fredholm et al., 1996a). (B) In human astrocytoma cells, D384 activation of protein kinase C is also able to enhance A_{2B} receptor-mediated cAMP accumulation, but the stimulation of calcium entry via the bradykinin B_2 receptor leads to inhibition of A_{2B} receptor-mediated cAMP accumulation (Altiok and Fredholm, 1993; Fredholm et al., 1996a).

pholipase C pathways potentiate cAMP accumulation stimulated by IP prostanoid receptors in HEL cells (Turner et al., 1992; Feoktistov et al., 1997). It has been suggested that protein kinase C does not exert its effect at the level of the receptor but rather affects the coupling of the stimulated G_s protein with adenyl cyclase (Fredholm, 1995). The synergistic interaction between the A_{2B} receptors and the calcium/protein kinase C pathway can occur further down in the signaling cascade. Thus, A_{2B} receptors greatly potentiate the phorbol 12-myristate 13-acetate-induced synthesis of IL-8 in human mast cells (Feoktistov and Biaggioni, 1995). In T-lymphocytes, the T-receptor is known to activate immediate early gene transcription, leading to the activation of the AP-1 transcription factor (Kvanta et al., 1992). A_{2B} receptors significantly potentiate this response, implying that cAMP, and calcium/protein kinase C pathways, may act in concert in the regulation of gene transcription (Kontny et al., 1992; Kvanta and Fredholm, 1994).

In summary, A_{2B} receptors are coupled to adenyl cyclase through G_s proteins in every cell studied. Current evidence suggests that the actions of A_{2B} receptors can be mediated not only by cAMP, but also by other intracellular pathways that may vary between cells. A_{2B} receptors can couple to calcium channels through G_s, but additional studies are needed to determine the type of channel involved. Similarly, it remains to be determined which member of the G_q family is responsible for A_{2B} receptor coupling to phospholipase C. It is of interest that, as far as intracellular pathways are concerned, A_{2B} receptors have as much in common with A_1 or A_3 receptors (activation of phospholipase C), as with A_{2A} receptors (activation of adenyl cyclase). It would be important to determine which domain of the A_{2B} receptor defines the differences in G protein coupling between A_{2A} and A_{2B} receptors.

VII. Physiological Functions of A_{2B} Recept rs

A. Control of Vascular Tone

Adenosine-induced vasodilation has been traditionally attributed to activation of A_{2A} receptors. However, the recent finding of the presence of A_{2B} receptors in some vascular beds raised the possibility that they participate in the regulation of vascular tone. Indeed, there are vascular beds in which the nonselective agonist NECA produces profound vasodilation, but the selective A_{2A} agonist CGS 21680 has little effect, suggesting that adenosine-induced vasodilation is mediated via A_{2B} receptors (for review, see Webb et al., 1992). This phenomenon is observed in guinea pig aorta and dog saphenous vein (Hargreaves et al., 1991), and in dog coronary arteries (Balwierczak et al., 1991). This effect is not due to species differences, because both A_{2A} and A_{2B} receptors may mediate vasodilation in the same species. In guinea pig, for instance, A_{2A} receptors mediate relaxation of coronary vessels, whereas A_{2B} receptors produce vasodilation of the aorta (Martin, 1992; Martin et al., 1993). Likewise, the A_{2A} agonist CGS 21680 lowers blood pressure in the intact dog (Levens et al., 1991), presumably by inducing vasodilation, despite its lack of efficacy in the coronary arteries of this species.

The vasodilatory effects of adenosine can be accounted for by a direct relaxing action on vascular smooth muscle cells. However, recent studies have suggested that the endothelium contributes to, or is even essential for, the vasodilatory effects of intravascular adenosine. It has been shown that most of the labeled adenosine administered intra-arterially is contained within endothelial cells, and very little escapes this endothelium trap to reach the underlying vascular smooth muscle (Nees et al., 1985). Similarly, intravascular administration of adenosine linked to macromolecules, and therefore less likely to cross the endothelium, is still able to produce vasodilation (Olsson et al., 1977).

In vitro studies, however, have yielded conflicting results as to whether the vasodilatory actions of adenosine are different in vascular preparation with intact or denuded endothelium (Rubanyi and Vanhoutte, 1985; Yen et al., 1988; Falcone et al., 1993; Maekawa et al., 1994).

Evaluation of a putative endothelium-dependent vasodilation by adenosine is challenging in ring preparation, because adenosine will produce vasodilation in preparations with or without endothelium. This is particularly true when stable agonists are used, because they are not trapped by the endothelium in the way adenosine is and have more ready access to the underlying vascular smooth muscle. Other endothelium-dependent vasodilators will constrict vascular smooth muscle in the absence of endothelium (Furchgott, 1984), making their distinction easier. Conversely, adenosine-induced vasodilation could conceivably produce flow-related release of nitric oxide (NO), giving the appearance of NO-mediated vasodilation (Olsson, 1996).

Methodological difficulties notwithstanding, more fundamental differences may explain the apparent discrepancies regarding the role of the endothelium on adenosine-induced vasodilation. Given the diversity of endothelial cell types, it is possible that endothelial vasodilatory responses to adenosine vary between species, and within the same species depending on the vascular bed being studied. It is unclear to what degree endothelial A_{2A} or A_{2B} receptors may contribute to these differences. This issue will be resolved only if studies that examine the endothelium-dependency of adenosine-induced vasodilation also define the adenosine receptor subtype involved.

A_{2B} receptors have been shown in endothelial cells. Both A_{2B} and A_{2A} receptors regulate cAMP production in human aortic (Iwamoto et al., 1994) and human umbilical vein (Feoktistov and Biaggioni, unpublished observations) endothelial cells, and A_{2B} receptor mRNA has been detected in human aortic endothelial cells (Iwamoto et al., 1994). Few studies have directly examined the possible interaction between A_{2B} receptors and endothelium-derived vasodilation, and results vary depending on the vascular bed studied. A_{2B} receptors mediate vasodilation in the rat mesenteric arterial bed (Rubino et al., 1995) and in the isolated blood-perfused rat lung preparation (Haynes et al., 1995). In both cases, A_{2B}-mediated vasodilation seems to be independent of NO generation, because they were not reversed by inhibition of NO synthase by N^G-nitro-L-arginine methyl ester (L-NAME) (Haynes et al., 1995; Rubino et al., 1995). On the contrary, isolated rat renal artery rings contain A_{2B} receptors that are located exclusively on the endothelium and cause NO release and vasodilation, because this vasodilation can be blocked with L-NAME and prevented by removal of the endothelium (Martin and Potts, 1994). Similarly, A_{2B} receptors also appear to vasodilate the rabbit corpus cavernosum, and this effect is reduced by removal of the endothelium (Chiang et al., 1994).

In summary, both A_{2A} and A_{2B} receptors mediate vasodilation. The relative contribution of A_{2B} receptors to adenosine-induced vasodilation is not defined. There are also conflicting results as to the importance of NO generation in adenosine-induced and A_{2B}-induced vasodilation, but there are some examples in which the endothelium contributes to A_{2B}-mediated vasodilation. To complicate things further, in some vascular beds, adenosine-induced vasodilation is endothelium-dependent but does not appear to be mediated by NO because it is not blocked by inhibition of NO synthase, raising the possibility that other endothelial factors, such as endothelium-dependent hyperpolarizing factor, may be involved (Headrick and Berne, 1990). The precise nature of the interaction between A_{2B} receptors and endothelial cells and their role in the regulation of vascular tone are areas where more research is needed.

B. Cardiac Myocyte Contractility

Adenosine has important protective effects against ischemia in the myocardium, but these effects are largely attributed to A_1 receptors (for review, see Olsson and Pearson, 1990). It has been reported recently that myocytes isolated from fetal chick ventricles, but not from the atria, possess functional A_{2B} and A_{2A} receptors. Both receptors are capable of augmenting myocardial contractility in this model. These adenosine effects, however, become evident only after inhibitory A_1 receptor pathways are inactivated with pertussis toxin (Liang and Haltiwanger, 1995). Presence of A_2 receptors, capable of stimulating cAMP accumulation, was demonstrated in cultured adult rodent myocardial cells after A_1 receptor blockade (Romano et al., 1989; Stein et al., 1993; Xu et al., 1996). These results could be explained by a possible contamination of myocardial preparations with fibroblasts and endothelial cells expressing A_{2B} receptors. However, studies performed on single cells argue against this possibility. A positive inotropic response mediated via A_2 receptors was demonstrated in cultured rat and guinea pig ventricular myocytes (Stein et al., 1993; Xu et al., 1996; Dobson and Fenton, 1997). The role of myocardial A_2 receptors in mediating a positive inotropic effect remains a controversial issue (Olsson, 1996), and their physiological significance is unclear, given that their effects become evident only under blockade of A_1 receptors.

C. Modulation of Neurosecretion and Neurotransmission

Adenosine is in general considered to be a depressor of neurons, inhibiting neurotransmitter release and other neuronal functions (Phillis et al., 1993a) and acts as a neuroprotective against ischemia (Dragunow and Faull, 1988). Many of these inhibitory actions are mediated by A_1 receptors (Dunwiddie and Fredholm, 1989). A_2 receptors, on the other hand, have been shown to mediate excitatory actions on the nervous system (Sebastião and Ribeiro, 1996). Earlier studies did not use specific agonists or antagonists to allow a precise identification of the A_2 receptor subtype involved, and relatively little information is available for the A_{2B} receptor. More re-

cently, several excitatory actions have been linked to the A_{2A} receptor, including enhancement of the release of several neurotransmitters, including acetylcholine, the excitatory amino acids glutamate and aspartate, dopamine, and norepinephrine (for review, see Sebastião and Ribeiro, 1996). However, gene knockout mice lacking A_{2A} receptors exhibit aggressive behavior and lack the stimulant effect of caffeine (Ledent et al., 1997), suggesting that A_{2A} receptors normally exert a tonic central depressant action. This is in agreement with several observations indicating a depressant effect of A_{2A} agonists on locomotor activity (Sebastião and Ribeiro, 1996). It should be noted, however, that comparisons between molecular mechanisms of excitation and integrated physiological responses need to be done with care. For example, adenosine depresses sympathetic nerve activity and blood pressure when injected into the nucleus tractus solitarii (Tseng et al., 1988) via activation of A_{2A} receptors (Barraco et al., 1991). This apparent depressant action, however, is mediated by local stimulation of the release of the excitatory amino acid glutamate (Mosqueda-Garcia et al., 1989, 1991), mediated by A_{2A} receptors (Castillo-Melendez et al., 1994).

A_{2B} receptors are widespread in the brain, but little is known about their function. There are, however, several examples of neuroexcitatory actions. Adenosine agonists increase the release of the excitatory amino acid aspartate in rat cerebral cortex cup superfusates in vivo while depressing the release of the inhibitory amino acid GABA (Phillis et al., 1993b). The agonist profile was suggestive of an A_{2B} receptor, in that it was produced by a high concentration of N^6-cyclopentyladenosine (CPA), but not by the A_{2A} agonist CGS 21680. If confirmed, these results would suggest that A_{2B} receptors would lead to greater tissue injury if activated during ischemia, an action that is in sharp contrast to the postulated protective effects of A_1 receptors. In this same model, A_{2B} receptors also enhance basal release of acetylcholine (Phillis et al., 1993a).

Acutely isolated pyramidal neurons from the CA3 region of guinea pig hippocampus contain A_1 receptors that inhibit N-type Ca^{2+} currents. After blockade of A_1 receptors, adenosine agonists potentiate a P-type Ca^{2+} current with a pharmacological profile consistent with A_{2B} receptors, inasmuch as the A_{2A} agonist CGS 21680 had no effect (Mogul et al., 1993). Likewise, after A_1 receptor blockade, A_{2B} receptors induce long-term potentiation in the CA1 region of rat hippocampus (Kessey et al., 1997). Although these studies were not designed to explore the site of action of A_{2B} receptors, the results were consistent with a postsynaptic site of action. It should be noted that the selective A_{2A} agonist CGS 21680 was also found to facilitate long-term potentiation in the hippocampal CA1 area (de Mendonca and Ribeiro, 1994), whereas A_1 receptors inhibit long-term potentiation in this area of the brain (Arai and Lynch, 1992). The relative importance of these contrasting pathways coupled to adenosine receptor subtypes remains to be defined under physiological and pathological conditions. Similarly, A_1 receptors inhibit dopamine release in rat striatum, but selective blockade of A_1 receptors reveals a stimulatory effect of A_{2B} receptors on dopamine release (Okada et al., 1996).

Adenosine also inhibits norepinephrine release from peripheral noradrenergic nerve terminals (Wakade and Wakade, 1978). This effect is thought to be mediated by putative presynaptic A_1 receptors (Paton, 1981), but the inhibition of norepinephrine release in isolated canine pulmonary arteries was better explained by A_{2B} receptors, based on rank order of potencies of agonists (Tamaoki et al., 1997). Similarly, the pharmacological profile for adenosine-induced inhibition of neurotransmission in rabbit corpus cavernosum is consistent with that of an A_{2B} receptor (Chiang et al., 1994). On the other hand, the nonselective A_2 agonist NECA potentiated acetylcholine release evoked by electrical stimulation of the rat bronchial smooth muscle and was 100-fold more potent than the A_{2A} agonist CGS 21680 (Walday and Aas, 1991). Furthermore, this process was blocked by 10 μM enprofylline. The effect of exogenous acetylcholine was not affected by NECA. Taken together, these results provide evidence for an A_{2B} presynaptic receptor that enhances neurally mediated release of acetylcholine and thereby induces contraction of bronchial smooth muscle (Walday and Aas, 1991).

Adenosine also modulates release of catecholamines from chromaffin cells. These adrenal medullary cells are under neural control through cholinergic nicotinic receptors. No specific binding was found using selective ligands for the A_1 or A_{2A} receptors in chromaffin cells from bovine adrenal medulla, but specific binding was obtained using [^{3}H]NECA. These results were interpreted as evidence that only A_{2B} receptors are expressed in these cells (Casado et al., 1992). At high (20 μM) concentrations, the nonselective agonist NECA inhibits catecholamine release induced by nicotinic stimulation, presumably by activation of A_{2B} receptors (Mateo et al., 1995). This effect has an unusually slow time course and is seen only after 20 to 30 minutes of preincubation with NECA, and its physiological relevance is not clear.

D. Cell Growth and Gene Expression

Whereas most studies of the cardiovascular effects of adenosine have focused on its acute actions on vascular tone and adrenergic neurotransmission, recent evidence suggests that adenosine may also play a long-term modulatory role in smooth muscle growth. Exogenous adenosine was shown to inhibit rat aortic smooth muscle cell growth induced by fetal calf serum, as assessed by a decrease in thymidine incorporation and in cell number (Dubey et al., 1996b). These inhibitory effects were reversed by the A_2 receptor antagonist KF 17837, but not by the A_1 antagonist DPCPX, and were not mimicked by the A_{2A} agonist CGS 21680, suggesting that this effect is

mediated by A_{2B} receptors (Dubey et al., 1996b). Activation of adenyl cyclase is postulated as the signaling pathway involved, because this effect is mimicked by 8-bromo-cAMP. These investigators later showed that stimulation of these vascular smooth muscle cells by fetal calf serum can trigger release of endogenous adenosine, which then acts in an autocrine fashion to inhibit growth. The importance of endogenous adenosine is less certain, because inhibition of vascular smooth muscle growth by endogenous adenosine is evidenced only if adenosine deaminase is inhibited (Dubey et al., 1996a). It is postulated that this reflects a limitation of the experimental model, due to the presence of adenosine deaminase in the fetal calf serum used to stimulate vascular smooth muscle growth. If a role for endogenous adenosine is confirmed, this finding would establish a novel cardioprotective effect of adenosine, with relevance to vascular remodeling process observed in hypertension and atherosclerosis.

A_{2B} receptors can also modulate gene expression, in some cases leading to inhibition of protein synthesis. For example, stimulation of A_{2B} receptors decreases collagenase gene expression in interleukin-1–stimulated cultured fibroblast-like synoviocytes, an effect apparently mediated by cAMP elevation (Boyle et al., 1996). In contrast, A_{2B} receptors promoted the synthesis of IL-8 in HMC-1 mast cells by a cAMP-independent mechanism (Feoktistov and Biaggioni, 1995). It has been recently demonstrated that A_{2B} receptors can also induce an increase in interleukin-6 mRNA levels and protein synthesis in the human astrocytoma cell line U373 MG (Fiebich et al., 1996a). The synergistic relationship between A_{2B} receptors and T-receptors, and generally between cAMP and protein kinase C pathways in gene expression, has been discussed in detail elsewhere (Fredholm, 1995; Fredholm et al., 1996a).

E. Regulation of Intestinal Tone and Secretion

The high levels of A_{2B} receptor expression found in different parts of the intestinal tract motivated great interest in defining their function. In some studies, A_{2B} receptor mRNA expression is greatest in intestinal tissue among all organs (Stehle et al., 1992). It appears that A_{2B} receptors may be involved in modulation of intestinal tone as well as intestinal secretion. Adenosine elicits relaxation of dispersed guinea pig longitudinal muscle cells from small intestine via A_{2B} receptors coupled to adenyl cyclase but produced contraction through A_1 receptors by increasing intracellular calcium (Murthy et al., 1995). The A_{2B}-mediated relaxation was evident only after A_1 receptor blockade, raising doubts as to their importance. However, blockade of A_2 receptors potentiated A_1-mediated contraction, indicating that A_{2B} receptors do provide a restraining function against intestinal contraction. In rat duodenum, A_{2B} receptors cause relaxation of longitudinal muscle but contraction of muscularis mucosae (Nicholls et al., 1996). This is an unexpected result and the first example of an excitatory response by A_{2B} receptors in a smooth muscle preparation. This tissue is also unusual in that A_1 receptors were found to produce relaxation of duodenal longitudinal muscle (Nicholls et al., 1996). A_{2B} receptors have also been shown to relax guinea pig taenia ceci, based on the pharmacological profile of agonists (Prentice and Hourani, 1997). The functional relevance of the intestinal relaxant actions of A_{2B} receptors has not been defined. Of interest, adenosine A_{2B} receptors also mediate relaxation of other visceral smooth muscle such as rat urinary bladder (Nicholls et al., 1996) and rat vas deferens (Hourani et al., 1993).

The effect of A_{2B} receptors on epithelial secretion has received particular attention because of its potential relevance to diarrheal processes. As part of the pathophysiology of these disorders, neutrophils are recruited into intestinal crypts, where they release a soluble "neutrophil-derived secretagogue" that then activates intestinal epithelium to stimulate chloride secretion. This chloride secretion has the net effect of producing isotonic fluid secretion, an important component of diarrheal diseases. This neutrophil-derived secretagogue has recently been identified as AMP (Madara et al., 1993), which is then converted to adenosine at the epithelial cell surface by ecto-5′-nucleotidase. It is adenosine that then acts as a paracrine mediator of chloride secretion (Madara et al., 1993). It was later demonstrated that neutrophil-derived adenosine elicits chloride secretion in the intestinal epithelial cell line T84 via activation of A_{2B} receptors (Strohmeier et al., 1995), implying the possible involvement of this receptor subtype in the pathophysiology of diarrheal diseases. In contrast to these findings, A_{2B} receptors reportedly inhibit intestinal fluid secretion induced by vasoactive intestinal peptide in rat jejunum (Hancock and Coupar, 1995). These results should be interpreted with caution, because receptor characterization was done by relative potency of agonists and antagonists injected intravenously in anesthetized rats. Definite receptor characterization cannot be completed until the tissue localization of these putative A_{2B} receptors is determined and in vitro studies are performed (Hancock and Coupar, 1995). The presence of A_{2B} receptors in epithelial cells of human intestine has been demonstrated by immunohistochemistry with an anti-A_{2B} receptor antibody (Puffinbarger et al., 1995), but more studies are needed to define their role in intestinal secretion and diarrheal processes.

F. Adenosine and Asthma

Adenosine has been implicated in the pathophysiology of asthma (for review, see Church and Holgate, 1986; Feoktistov and Biaggioni, 1996), and several lines of evidence support this hypothesis. Inhaled adenosine, or its precursor AMP, provokes bronchoconstriction in asthmatic patients (Cushley et al., 1984). This effect is fairly specific for patients with asthma, and even high

concentrations of inhaled adenosine fail to produce bronchoconstriction in the majority of normal subjects. Atopic subjects appear to be more responsive to inhaled AMP than they are to methacholine (Phillips et al., 1990), suggesting that adenosine may be a better discriminator of the disease. This preferential bronchoconstrictor effect in asthmatics is also observed with intravenous administration of adenosine (Drake et al., 1994) and in isolated human bronchi (Björck et al., 1992). Dipyridamole, a drug that blocks adenosine uptake and increases its extracellular concentrations, can also produce severe bronchospasm in asthmatic patients (Eagle and Boucher, 1989). Moreover, theophylline provides a better protection against adenosine-induced bronchoconstriction than against histamine-induced bronchoconstriction (Mann and Holgate, 1985).

The mechanism by which adenosine produces bronchoconstriction has been the focus of recent interest. In particular, it would be important to define the receptor type involved. Adenosine produces a direct constrictor action on isolated guinea pig trachea with an agonist profile consistent with A_1 receptors (Ghai et al., 1987), and this process is not blocked by enprofylline (Farmer et al., 1988). In this same preparation, however, A_2 receptors were found to produce relaxation, but the receptor subtype was not identified. Based on rank order of potencies for agonists, it was found that A_1 receptors also mediate bronchoconstriction in an allergic rabbit model in vivo (Ali et al., 1994a,b), and treatment with antisense oligodeoxynucleotide targeting the adenosine A_1 receptor desensitized the allergic rabbits to subsequent challenge with either adenosine or allergen (Nyce and Metzger, 1997). Adenosine also constricts human bronchi isolated from asthmatics in vitro but not bronchi isolated from nonasthmatics (Björck et al., 1992). The contractile effect of adenosine was inhibited with 2-thio-[(1,3-dipropyl)-8-cyclopentyl]-xanthine, and this was taken as evidence of an A_1-mediated effect.

The bronchoconstriction produced by inhaled adenosine in humans appears to be mediated through mast cell activation, because it can be blocked by specific antihistamines (Phillips et al., 1987; Rafferty et al., 1987) and prevented by cromoglycate and nedocromil sodium, drugs that inhibit mast cell degranulation (Phillips et al., 1989). Furthermore, a significant rise in plasma levels of histamine is detected after AMP challenge (Phillips et al., 1990). More recently, inhaled adenosine has been shown to increase levels of histamine, PGD_2, and tryptase in bronchoalveolar lavage fluid from asthmatics but not from nonasthmatics (Polosa et al., 1995). Tryptase is a highly specific marker for mast cells (Schwartz, 1990) and provides strong evidence that these cells are activated by adenosine in vivo.

In summary, exogenous adenosine provokes asthma, potentiation of endogenous adenosine with dipyridamole also produces bronchoconstriction, and blockade of endogenous adenosine with theophylline is helpful in preventing asthma. The bronchoconstriction induced by inhaled adenosine is unique to asthmatics and not observed in nonasthmatics. Current evidence suggests that this phenomenon involves mast cell activation. It is important, therefore, to elucidate the adenosine receptor subtype that mediates this phenomenon.

G. Adenosine Receptors and Mast Cells

Marquardt et al. (1978) were the first to report that adenosine, although ineffective alone, potentiated histamine release induced by anti-immunoglobulin E (IgE), concanavalin A, compound 48/80, or the calcium ionophore A23187 in isolated rat mast cells. The mechanisms that mediate potentiation of these cells remain unclear. Stimulation of adenyl cyclase by adenosine was blocked by methylxanthines, but potentiation of histamine release was not, suggesting that these effects were mediated by different adenosine receptors (Church et al., 1986).

Because potentiation of rat peritoneal mast cells is insensitive to methylxanthines, the possibility was raised that this effect is mediated by A_3 receptors, because the rat A_3 receptor has remarkably low affinity for methylxanthines (Zhou et al., 1992). This possibility was examined in the rat basophilic leukemia cell line RBL 2H3, which has been used as a model for rat mast cells. Adenosine analogs stimulated phospholipase C, increased cytoplasmic calcium, and potentiated mediator release in these cells with a pharmacological profile consistent with A_3 receptors (Ramkumar et al., 1993). Expression of A_3 receptors in RBL 2H3 cells was confirmed by radioligand binding and detection of mRNA (Ramkumar et al., 1993). The effects mediated by A_3 receptors in RBL 2H3 were blocked by pertussis toxin, suggesting a role of G_i-derived $\beta\gamma$ subunits in the activation of β-phospholipase C. Coupling of A_3 receptors to G_{i2} and G_{i3} proteins was recently reported (Palmer and Stiles, 1995). Of interest, A_{2A} and A_{2B}, but not A_1 receptors, have also been found in RBL 2H3 cells (Marquardt et al., 1994; Ramkumar et al., 1995); however, their function has not been elucidated. It should also be noted that A_1 receptors, to our knowledge, have not been found in other mast cell types (Marquardt et al., 1994).

It is important to consider that mast cells from different species, and even from different anatomical sites within the same species, can vary substantially in their morphological and biochemical characteristics and their response to pharmacological agents. There is increasing evidence that A_{2B} receptors modulate mast cell function. Adenosine activates adenyl cyclase and protein kinase C and potentiates mediator release in mouse bone marrow-derived mast cells (Marquardt and Walker, 1990). It appears that the ability of adenosine to activate protein kinase C and thereby to augment mast cell degranulation are independent of changes in cAMP (Marquardt and Walker, 1994). Both A_{2A} and A_{2B} transcripts were detected in mouse bone marrow-derived mast cells (Mar-

quardt et al., 1994). The failure of the A_{2A}-specific agonist CGS 21680 to enhance mediator release suggests that A_{2B} receptors modulate degranulation of these mast cells (Marquardt et al., 1994).

A_{2B} receptors have been shown to activate the human mast cell line HMC-1 (Feoktistov and Biaggioni, 1995). HMC-1 cells were derived from a patient with mast cell leukemia and their neutral proteases content is similar to that of human lung mast cells. These cells coexpress A_{2A} and A_{2B} receptors, and both are coupled to adenyl cyclase through G_s proteins. However, only A_{2B} receptors activate HMC-1 cells, as indicated by stimulation of IL-8 secretion. Furthermore, this effect was not mediated by cAMP, but by coupling to phospholipase C through a cholera toxin- and pertussis toxin-insensitive G protein, presumably of the G_q family (Feoktistov and Biaggioni, 1995). A_{2B} receptors not only produced direct stimulation of HMC-1 cells, but also potentiated phorbol 12-myristate 13-acetate-stimulated secretion of IL-8 (Feoktistov and Biaggioni, 1995). The expression of A_{2B} receptors in HMC-1 cells was recently confirmed by immunoblotting and fluorescent immunostaining with a specific anti-A_{2B} antibody (Feoktistov et al., 1996). Virtually identical findings have been reported in a canine BR mastocytoma cell line. Stimulation of A_{2B}, but not A_3 receptors, directly increased β-hexosaminidase release and also potentiated A23187-induced degranulation of mast cells. Also, these effects were not blocked by pertussis toxin (Auchampach et al., 1996).

In parenchymal human lung mast cells, obtained from normal sections of surgical specimens, adenosine does not directly evoke release of histamine and LTC_4, but in micromolar concentrations it potentiates mediator release from immunologically activated cells (Peachell et al., 1991). The order of potency of adenosine analogs and the low affinity of this process suggests that the response of human lung mast cells to adenosine is mediated by A_{2B} receptors. In support for this notion, the presence of A_{2B} has been recently demonstrated in bronchoalveolar lavage mast cells by double immunostaining with specific anti-A_{2B} and antitryptase antibodies (Feoktistov et al., 1996).

Given that inhaled adenosine affects only asthmatics but has no effect in nonasthmatics, there appears to be an intrinsic difference in the way adenosine interacts with mast cells from asthmatics. The in vitro response produced by A_{2B} receptors in HMC-1 cells and in canine BR mastocytoma cells appears to mimic the in vivo responses to inhaled adenosine in asthmatics, inasmuch as adenosine provokes mast cells activation in these cell lines as it does in asthmatics. On the other hand, the in vitro response of mast cells from normal human lung to adenosine resembles the effect of A_{2B} receptors in mouse bone marrow-derived mast cells, because in both cases adenosine potentiates mast cells activation but does not evoke direct activation. The molecular mechanisms behind these differential A_{2B}-mediated responses in asthmatic versus normal mast cells, and in HMC-1 cells versus mouse bone marrow-derived mast cells, remain unknown.

Despite the direct bronchoconstricting action of A_1 receptors observed in allergic rabbits (Nyce and Metzger, 1997), the bronchoconstriction induced by inhaled adenosine in humans is better explained by an A_{2B} receptor. Adenosine-induced bronchoconstriction appears to be mediated by mast cell activation, and A_1 receptors have not been described in mast cells, whereas A_{2B} receptors are expressed in human mast cells. Moreover, enprofylline is an effective antiasthmatic and is a selective A_{2B} blocker at concentrations achieved clinically. Finally, A_{2B} receptors have been shown to potentiate neurally mediated cholinergic bronchoconstriction through an enprofylline-sensitive process (Walday and Aas, 1991). The evidence presented so far does not preclude the contribution of more than one adenosine receptor in asthma, or the possibility that nonadenosinergic mechanisms contribute to the antiasthmatic effects of enprofylline and other methylxanthines.

VIII. A_{2B} Receptors as Therapeutic Targets

The ability of adenosine to delay atrioventricular node conduction was the basis for its development as a therapeutic agent in the treatment of supraventricular arrhythmias (Belardinelli et al., 1989) and has become the drug of choice for the termination of that arrhythmia. Taking advantage of its profound vasodilatory effects, intravenous adenosine is used as a stress test in the diagnosis of myocardial ischemia (Verani et al., 1990). Adenosine was investigated as a hypotensive agent during anesthesia (Sollevi et al., 1984), where the reflex sympathetic activation induced by this agent (Biaggioni, 1992) is not observed. However, adenosine has been implicated in many other physiological and pathological processes. The biggest problem in translating this knowledge into therapeutic tools is perhaps the ubiquity of adenosine receptors, which often mediate contrasting effects. The challenge is how to develop drugs that will selectively target a receptor mediating a specific action. The ongoing development of selective agonists or antagonist represents a substantial advancement toward this goal. Nonetheless, even if specific agents can be developed for a given receptor subtype, the problem remains of selectively targeting the site of action. For example, A_1-selective agonists could be developed for their antilipolytic potential. If given systemically, however, it is possible that atrioventricular conduction delay or bradycardia may be an undesirable, and perhaps limiting effect, given that these actions are also mediated by A_1 receptors. In the development of useful therapeutic agents, therefore, care should be taken not only in the targeting of the receptor subtype, but also the site of action. This problem, of course, is not limited to adenosinergic systems and is common to others characterized by the widespread nature of their receptors.

Given that the functional role of A_{2B} receptors is only now being addressed, a discussion of potential therapeutic opportunities arising from modulation of such receptors is necessarily speculative. There are, however, some promising areas that deserve further attention. The potential role of A_{2B} receptors in asthma can be used as an example. If confirmed, this mechanism would provide a novel approach for the treatment of this condition. Asthma continues to be a substantial medical problem that affects approximately 5 to 7% of the population. Despite advances in its treatment, the prevalence of asthma, emergency department visits, hospitalizations, and mortality related to the disease all appear to be on the rise (Gergen et al., 1988; Vollmer et al., 1992; Weiss et al., 1993). Theophylline continues to be an effective treatment in the prevention of asthma attacks, more than as an acute bronchodilator (Weinberger and Hendeles, 1996), but considerable plasma levels, in the range of 20–80 μM, are needed for it to be effective. Moreover, it has many side effects, which can be attributed to its nonspecificity. Its central actions contribute to theophylline's side-effect profile and are of doubtful benefit for the treatment of asthma.

If indeed blockade of A_{2B} receptors contributes to the antiasthmatic effects of theophylline, it would be possible to develop selective antagonists for this receptor subtype. Lipophobic compounds would have the advantage of not crossing the blood-brain barrier. Specific targeting to the site of action can also be accomplished if compounds that can be administered by inhalation are developed. This proposition is not unrealistic. For example, the xanthine antagonist DPSPX is approximately 100-fold more potent than theophylline as an A_{2B} receptor antagonist. Because of a charge moiety in its molecule, this water-soluble xanthine does not penetrate cell membranes or cross the blood-brain barrier (Tofovic et al., 1991). It appears that the ionic p-sulfophenyl substituent in DPSPX may confer high A_{2B} potency. The lack of L-alkyl substituents in the enprofylline molecule renders it an ineffective antagonist of other adenosine receptor subtypes. The systematic study of the structure-activity relationship for blockade of A_{2B} receptors, considering the above-mentioned properties, could result in more potent and specific agents. Similarly, A_{2B} receptor antagonists can be developed for the treatment of diarrheal processes, if adenosine is confirmed to play a role in this process. Targeting to the site of action could be achieved with compounds that are poorly absorbed, as long as they are able to reach the intestinal crypts involved in intestinal inflammatory processes.

Development of agonists to target A_{2B} receptors, for example, to inhibit vascular smooth muscle growth, would be a greater challenge. Substantial progress would need to be made to develop an agonist potent enough to selectively activate the low-affinity A_{2B} receptor while having negligible actions at other receptors. The actions of endogenous adenosine could be enhanced with uptake inhibitors, or adenosine-regulating agents (Mullane, 1993), but this approach would activate all adenosine receptors, perhaps others even more than A_{2B} receptors. Targeting the site of action is an additional problem that would have to be resolved for any drug that had to be administered systemically. The observation that A_{2B} receptors mediate vasodilation of the rabbit corpus cavernosum (Chiang et al., 1994) raises the possibility that an agonist to this receptor type can be useful in impotence. Direct injections of adenosine into the corpus cavernosum of impotence patients produce a brief erection (Kilic et al., 1994), particularly if combined with prostaglandin E_1 (Chiang et al., 1994). The short duration of effect is clearly related to the short half-life of adenosine in humans (Moser et al., 1989). If this effect is also mediated by A_{2B} receptors in humans, it will be possible to develop stable and selective agonists that can be given locally.

IX. Concluding Remarks

Until recently, relatively little attention has been paid to A_{2B} receptors. It is instructive that contemporary reviews about adenosine only briefly mention their existence. Conclusions about the selective nature of agonists or antagonists at specific adenosine receptors have often been made without testing these compounds on A_{2B} receptors. The A_{2B} receptor was often assumed to be simply a low-affinity variant of the A_{2A} receptor. A_{2A} and A_{2B} receptors are frequently found in the same tissue, and, because both were thought to act by adenyl cyclase activation, it is easy to assume that A_{2B} receptors will be of lesser physiological significance. The lack of selective pharmacological probes with which to study this receptor subtype has been the main obstacle in defining the role of A_{2B} receptors.

Several factors can explain the increasing interest in A_{2B} receptors. The cloning of adenosine receptors validated the belief that A_{2B} receptors were distinct from A_{2A} receptors. It also revealed its widespread and distinct distribution in tissues. The development of selective agonists and antagonists for other adenosine receptor types has indirectly improved our knowledge of A_{2B} receptors, but their pharmacological characterization is still mostly done by a method of exclusion, i.e., by the lack of efficacy of agonists and antagonists that are selective at other receptors. Also, other tools now are available for the study of this receptor. The amino acid sequence of the receptor, the nucleotide sequence encoding the receptor, and genomic information are now available. This opens the door for mutational or chimeric analysis of A_{2B} receptors to understand the molecular determinants for its unique coupling to G proteins. It is also possible to determine with precision the cellular localization of A_{2B} receptors, particularly now that antibodies against this receptor have been generated.

Significant progress has been made using currently available tools. It is now clear that, in addition to adenyl

cyclase, A_{2B} receptors can also couple to other intracellular pathways, including calcium channels and phospholipase C. In that sense, A_{2B} receptors have as much in common with A_1 and A_3 receptors, as with A_{2A} receptors. The functional relevance of A_{2B} receptors is being defined, but much work is needed in this area. Given the ubiquitous nature of adenosine receptors, it is not surprising that more than one adenosine subtype is found in the same tissue, but it is now evident that they can be coexpressed in the same cell. In these situations, it would seem that the functional role of higher-affinity receptors would predominate over A_{2B} receptors. Nonetheless, A_{2B} receptors can play a role under these conditions by modulating events triggered by other receptor systems. Examples have been presented in this review of A_{2B} receptors restraining the actions of A_1 receptors, or potentiating the effects of thrombin and T-cell receptors. The relative importance of A_{2B} receptors may be greater in situations characterized by substantial increases in interstitial levels of adenosine, as occurs during ischemia in metabolically active tissues. On the other hand, there are cellular systems in which the actions of A_{2B} receptors appear to predominate. Such is the case, for example, of human mast cells, epithelial intestinal cells, and the regulation of vascular smooth muscle growth, among others.

Greater advances can now be made by the simple appreciation of the unique features of this receptor type. In the past, investigators often have failed to realize the potential involvement of A_{2B} receptors in their experimental systems. However, the study of A_{2B} receptors will be considerably improved by the introduction of specific pharmacological probes for this receptor type. Even though adenosine analogs have, in general, a poor affinity for A_{2B} receptors, this is not the case for antagonists. Therefore, development of potent and selective A_{2B} antagonists appears particularly promising and likely will further our knowledge of A_{2B} receptors. Specific antagonists will be particularly useful in defining the role of A_{2B} receptors in physiological and pathological situations. The appreciation of the potential role of A_{2B} receptors in the pathogenesis of disease processes, including asthma, vascular remodeling, and diarrhea, raises the possibility that A_{2B} receptors may become the target for future drug development.

Acknowledgments. The authors would like to thank Drs. Jack N. Wells and Lee Limbird, Department of Pharmacology, Vanderbilt University, for helpful discussions in the preparation of this manuscript. The authors would also like to thank the reviewers for their constructive suggestions.

REFERENCES

AATSINKI, J. T., PIETILA, E. M., LAKKAKORPI, J. T., AND RAJANIEMI, H. J.: Expression of the LH/CG receptor gene in rat ovarian tissue is regulated by an extensive alternative splicing of the primary transcript. Mol. Cell. Endocrinol. **84:** 127–135, 1992.

ALEXANDER, S. P. H., COOPER, J., SHINE, J., AND HILL, S. J.: Characterization of the human brain putative A_{2B} receptor expressed in Chinese hamster ovary (CHO) A_{2B4} cells. Br. J. Pharmacol. **119:** 1286–1290, 1996.

ALI, S., MUSTAFA, S. J., AND METZGER, W. J.: Adenosine-induced bronchoconstriction and contraction of airway smooth muscle from allergic rabbits with late-phase airway obstruction: evidence for an inducible adenosine A1 receptor. J. Pharmacol. Exp. Ther. **94:** 1328–1334, 1994a.

ALI, S., MUSTAFA, S. J., AND METZGER, W. J.: Adenosine receptor-mediated bronchoconstriction and bronchial hyperresponsiveness in allergic rabbit model. Am. J. Physiol. **94:** L271–L277, 1994b.

ALTIOK, N., BALMFORTH, A. J., AND FREDHOLM, B. B.: Adenosine receptor-induced cAMP changes in D384 astrocytoma cells and the effect of bradykinin thereon. Acta Physiol. Scand. **144:** 55–63, 1992.

ALTIOK, N., AND FREDHOLM, B. B.: Bradykinin inhibits cyclic AMP accumulation in D384 human astrocytoma cells via a calcium-dependent inhibition of adenylyl cyclase. Cell. Signalling **5:** 279–288, 1993.

AMATRUDA, T. T., III, STEELE, T. D., SLEPAK, V. Z., AND SIMON, M. I.: $G\alpha_{15}$, a G protein alpha subunit specifically expressed in hematopoietic cells. Proc. Natl. Acad. Sci. USA **88:** 5587–5591, 1991.

ARAI, A., AND LYNCH, G.: Factors regulating the magnitude of long-term potentiation induced by theta pattern stimulation. Brain Res. **598:** 173–184, 1992.

AUCHAMPACH, J. A., CAUGHEY, G. H., AND LINDEN, J.: A_{2B} and not A_3 adenosine receptors mediate degranulation of canine BR mastocytoma cells. (Abstract) Drug Dev. Res. **37:** 147, 1996.

BALWIERCZAK, J. L., SHARIF, R., KRULAN, C. M., FIELD, F. P., WEISS, G. B., AND MILLER, M. J.: Comparative effects of a selective adenosine A2 receptor agonist, CGS 21680, and nitroprusside in vascular smooth muscle. Eur. J. Pharmacol. **196:** 117–123, 1991.

BARRACO, R. A., EL-RIDI, M. R., ERGENE, E., AND PHILLIS, J. W.: Adenosine receptor subtypes in the brainstem mediate distinct cardiovascular response patterns. Brain Res. Bull. **26:** 59–84, 1991.

BELARDINELLI, L., LINDEN, J., AND BERNE, R. M.: The cardiac effects of adenosine. Prog. Cardiovasc. Dis. **32:** 73–97, 1989.

BELARDINELLI, L., SHRYOCK, J. C., RUBLE, J., MONOPOLI, A., DIONISOTTI, S., ONGINI, E., DENNIS, D. M., AND BAKER, S. P.: Binding of the novel nonxanthine A_{2A} adenosine receptor antagonist [^{3}H]SCH 58261 to coronary artery membranes. Circ. Res. **79:** 1153–1160, 1996.

BIAGGIONI, I.: Contrasting excitatory and inhibitory effects of adenosine in blood pressure regulation. Hypertension **20:** 457–465, 1992.

BJÖRCK, T., GUSTAFSSON, L. E., AND DAHLÉN, S. E.: Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. Am. Rev. Respir. Dis. **145:** 1087–1091, 1992.

BLAZYNSKI, C.: Characterization of adenosine A_2 receptors in bovine retinal pigment epithelial membranes. Exp. Eye Res. **56:** 595–599, 1993.

BOYLE, D. L., SAJJADI, F. G., AND FIRESTEIN, G. S.: Inhibition of synoviocyte collagenase gene expression by adenosine receptor stimulation. Arthritis Rheum. **39:** 923–930, 1996.

BRACKETT, L. E., AND DALY, J. W.: Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. Biochem. Pharmacol. **47:** 801–814, 1994.

BRUNS, R. F.: Adenosine antagonism by purines, pteridines and benzopteridines in human fibroblasts. Biochem. Pharmacol. **30:** 325–333, 1981.

BRUNS, R. F., FERGUS, J. H., BADGER, E. W., BRISTOL, J. A., SANTAY, L. A., HARTMAN, J. D., HAYS, S. J., AND HUANG, C. C.: Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. Naunyn-Schmiedeberg's Arch. Pharmacol. **335:** 59–63, 1987.

BRUNS, R. F., LU, G. H., AND PUGSLEY, T. A.: Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol. Pharmacol. **29:** 331–346, 1986.

BURNSTOCK, G.: A basis for distinguishing two types of purinergic receptors. *In* Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach, ed. by R. W. Straub and L. Bolis, pp. 107–118, Raven Press, New York, 1978.

CASADO, V., CASILLAS, T., MALLOL, J., CANELA, E. I., LLUIS, C., AND FRANCO, R.: The adenosine receptors present on the plasma membrane of chromaffin cells are of the A_{2b} subtype. J. Neurochem. **59:** 425–431, 1992.

CASTILLO-MELENDEZ, M., KRSTEW, E., LAWRENCE, A. J., AND JARROTT, B.: Presynaptic adenosine A_{2a} receptors on soma and central terminals of rat vagal afferent neurons. Brain Res. **94:** 137–144, 1994.

CHERN, Y., LAI, H. L., FONG, J. C., AND LIANG, Y.: Multiple mechanisms for desensitization of A2a adenosine receptor-mediated cAMP elevation in rat pheochromocytoma PC12 cells. Mol. Pharmacol. **44:** 950–958, 1993.

CHIANG, P. H., WU, S. N., TSAI, E. M., WU, C. C., SHEN, M. R., AND HUANG, C. H.: Adenosine modulation of neurotransmission in penile erection. Br. J. Clin. Pharmacol. **38:** 357–362, 1994.

CHURCH, M. K., AND HOLGATE, S. T.: Adenosine and asthma. Trends Pharmacol. Sci. **7:** 49–50, 1986.

CHURCH, M. K., HUGHES, P. J., AND VARDEY, C. J.: Studies on the receptor mediating cyclic AMP-independent enhancement by adenosine by IgE-dependent mediator release from rat mast cells. Br. J. Pharmacol. **87:** 233–242, 1986.

CUSHLEY, M. J., TATTERSFIELD, A. E., AND HOLGATE, S. T.: Adenosine-induced bronchoconstriction in asthma. Am. Rev. Respir. Dis. **129:** 380–384, 1984.

DALY, J. W.: The nature of receptors regulating the formation of cyclic AMP in brain tissue. Life Sci. **18:** 1349–1358, 1976.

DALY, J. W., BUTTS-LAMB, P., AND PADGETT, W.: Subclasses of adenosine

receptors in the central nervous system: interaction with caffeine and related methylxanthines. Cell. Mol. Neurobiol. **3:** 69–80, 1983.

DALZIEL, H. H., AND WESTFALL, D. P.: Receptors for adenine nucleotides and nucleosides: subclassification, distribution, and molecular characterization. Pharmacol. Rev. **46:** 449–466, 1994.

DAVAL, J.-L., NICOLAS, F., AND DORIAT, J.-F.: Adenosine physiology and pharmacology: how about A_2 receptors? Pharmacol. Ther. **71:** 325–335, 1996.

DELL, K. R., AND WILLIAMS, L. T.: A novel form of fibroblast growth factor receptor 2: alternative splicing of the third immunoglobulin-like domain confers ligand binding specificity. J. Biol. Chem. **267:** 21225–21229, 1992.

DE MENDONCA, A., AND RIBEIRO, J. A.: Endogenous adenosine modulates long-term potentiation in the hippocampus. Neuroscience **62:** 385–390, 1994.

DIONISOTTI, S., FERRARA, S., MOLTA, C., ZOCCHI, C., AND ONGINI, E.: Labeling of A_{2A} adenosine receptors in human platelets by use of the new nonxanthine antagonist radioligand [^{3}H]SCH 58261. J. Pharmacol. Exp. Ther. **278:** 1209–1214, 1996.

DIXON, K. D., GUBITZ, A. K., SIRINATHSINGHJI, D. J. S., RICHARDSON, P. J., AND FREEMAN, T. C.: Tissue distribution of adenosine receptor mRNAs in the rat. Br. J. Pharmacol. **118:** 1461–1468, 1996.

DOBSON, J. G., JR., AND FENTON, R. A.: Adenosine A_2 receptor function in rat ventricular myocytes. Cardiovasc. Res. **34:** 337–347, 1997.

DRAGUNOW, M., AND FAULL, R. L. M.: Neuroprotective effects of adenosine. Trends Pharmacol. Sci. **9:** 193–194, 1988.

DRAKE, I., ROUTLEDGE, P. A., AND RICHARDS, R.: Bronchospasm induced by intravenous adenosine. Hum. & Exp. Toxicol. **13:** 263–265, 1994.

DRURY, A. W., AND SZENT-GYÖRGYI, A.: The physiological activity of adenosine compounds with a special reference to their action upon the mammalian heart. J. Physiol. **68:** 213–237, 1929.

DUBEY, R. K., GILLESPIE, D. G., MI, Z., SUZUKI, F., AND JACKSON, E. K.: Smooth muscle cell-derived adenosine inhibits cell growth. Hypertension **27:** 766–773, 1996a.

DUBEY, R. K., GILLESPIE, D. G., OSAKA, K., SUZUKI, F., AND JACKSON, E. K.: Adenosine inhibits growth of rat aortic smooth muscle cells: possible role of A_{2b} receptor. Hypertension **27:** 786–793, 1996b.

DUNWIDDIE, T. V., AND FREDHOLM, B. B.: Adenosine A_1 receptors inhibit adenylate cyclase activity and neurotransmitter release and hyperpolarize pyramidal neurons in rat hippocampus. J. Pharmacol. Exp. Ther. **249:** 31–37, 1989.

EAGLE, K. A., AND BOUCHER, C. A.: Intravenous dipyridamole infusion causes severe bronchospasm in asthmatic patients. (Editorial) Chest **95:** 258–289, 1989.

ELFMAN, L., LINDGREN, E., WALUM, E., AND FREDHOLM, B. B.: Adenosine analogues stimulate cyclic AMP-accumulation in cultured neuroblastoma and glioma cells. Acta Pharmacol. Toxicol. **55:** 297–302, 1984.

FALCONE, J. C., KUO, L., AND MEININGER, G. A.: Endothelial cell calcium increase during flow-induced dilation in isolated arterioles. Am. J. Physiol. **264:** H653–H659, 1993.

FARMER, S. G., CANNING, B. J., AND WILKINS, D. E.: Adenosine receptor-mediated contraction and relaxation of guinea-pig isolated tracheal smooth muscle: effects of adenosine antagonists. Br. J. Pharmacol. **95:** 371–378, 1988.

FEOKTISTOV, I., AND BIAGGIONI, I.: Characterization of adenosine receptors in human erythroleukemia cells: further evidence for heterogeneity of adenosine A_2 receptors. Mol. Pharmacol. **43:** 909–914, 1993.

FEOKTISTOV, I., AND BIAGGIONI, I.: Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells: an enprofylline-sensitive mechanism with implications for asthma. J. Clin. Invest. **96:** 1979–1986, 1995.

FEOKTISTOV, I., AND BIAGGIONI, I.: Role of adenosine in asthma. Drug Dev. Res. **39:** 333–336, 1996.

FEOKTISTOV, I., AND BIAGGIONI, I.: Pharmacological characterization of adenosine A_{2B} receptors: studies in human mast cells co-expressing A_{2A} and A_{2B} adenosine receptor subtypes. Biochem. Pharmacol., in press, 1997.

FEOKTISTOV, I., BREYER, R. M., AND BIAGGIONI, I.: Prostanoid receptor with a novel pharmacolgical profile in human erythroleukemia cells. Biochem. Pharmacol. 54: 917–926, 1997.

FEOKTISTOV, I., MURRAY, J. J., AND BIAGGIONI, I.: Positive modulation of intracellular Ca^{2+} by adenosine A_{2b}, prostacyclin and prostaglandin E_1 receptors via a cholera toxin-sensitive mechanism in human erythroleukemia cells. Mol. Pharmacol. **45:** 1160–1167, 1994.

FEOKTISTOV, I., SHELLER, J. R., AND BIAGGIONI, I.: Adenosine A_{2b} receptors in human lung mast cells as a target for antiasthmatic methylxanthines. (Abstract) FASEB J. **10:** A1232, 1996.

FIEBICH, B. L., BIBER, K., GUYFKO, K., BERGER, M., BAUER, J., AND VAN CALKER, D.: Adenosine A_{2b} receptors mediate an increase in interleukin (IL)-6 mRNA and IL-6 protein synthesis in human astroglioma cells. J. Neurochem. **66:** 1426–1431, 1996a.

FIEBICH, B. L., BIBER, K., LIEB, K., VAN CALKER, D., BERGER, M., AND GEBICKE-HAERTER, P. J.: Cycooxygenase-2 expression in rat microglia is induced by adenosine A_{2A} receptors. Glia **18:** 152–160, 1996b.

FREDHOLM, B. B.: Purinoceptors in the nervous system. Pharmacol. Toxicol. **76:** 228–239, 1995.

FREDHOLM, B. B., ABBRACCHIO, M. P., BURNSTOCK, G., DALY, J. W., HARDEN, T. K., JACOBSON, K. A., LEFF, P., AND WILLIAMS, M.: Nomenclature and classification of purinoceptors. Pharmacol. Rev. **46:** 143–156, 1994.

FREDHOLM, B. B., ABBRACCHIO, M. P., BURNSTOCK, G., DUBYAK, G. R., HARDEN, T. K., JACOBSON, K. A., SCHWABE, U., AND WILLIAMS, M.: Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol. Sci. **18:** 79–82, 1997.

FREDHOLM, B. B., ARSLAN, G., KULL, B., KONTNY, E., AND SVENNINGSSON, P.: Adenosine (P1) receptor signaling. Drug Dev. Res. **39:** 262–268, 1996a.

FREDHOLM, B. B., BURNSTOCK, G., HARDEN, T. K., AND SPEDDING, M.: Receptor nomenclature. Drug Dev. Res. **39:** 461–466, 1996b.

FREDHOLM, B. B., LINDGREN, E., LINDSTRÖM, K., AND NORSTEDT, C.: Alpha-adrenoreceptor stimulation, but not muscarinic stimulation, increases cyclic AMP accumulation in brain slices due to protein kinase C mediated enhancement of adenosine receptor effects. Acta Physiol. Scand. **131:** 543–551, 1987.

FREDHOLM, B. B., AND PERSSON, C. G.: Xanthine derivatives as adenosine receptor antagonists. Eur. J. Pharmacol. **81:** 673–676, 1982.

FREDHOLM, B. B., AND SANDBERG, G.: Inhibition by xanthine derivatives of adenosine receptor-stimulated cyclic adenosine 3'-5'-monophosphate accumulation in rat and guinea-pig thymocytes. Br. J. Pharmacol. **80:** 639–644, 1983.

FREDHOLM, B. B., ZHANG, Y., AND VAN DER PLOEG, I.: Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. Naunyn-Schmiedeberg's Arch. Pharmacol. **354:** 262–267, 1996c.

FURCHGOTT, R. F.: The role of endothelium in the responses of vascular smooth muscle to drugs. Ann. Rev. Pharmacol. Toxicol. **24:** 175–197, 1984.

GALLO-RODRIGUEZ, C., JI, X.-D., MELMAN, N., SIEGMAN, B. D., SANDERS, L. H., ORLINDA, J., FISCHER, B., PU, Q., OLAH, M. E., VAN GALEN, P. J. M., STILES, G. L., AND JACOBSON, K. A.: Structure-activity relationship of N^6-Benzyladenosine-5'-uronamides as A_3-selective adenosine agonists. J. Med. Chem. **37:** 636–646, 1994.

GERGEN, P. J., MULLALLY, D. I., AND EVANS, R., III.: National survey of prevalence of asthma among children in the United States, 1976 to 1980. Pediatrics **81:** 1–7, 1988.

GERWINS, P., AND FREDHOLM, B. B.: Glucocorticoid receptor activation leads to up-regulation of adenosine A_1 receptors and down-regulation of adenosine A_2 responses in DDT_1 MF-2 smooth muscle cells. Mol. Pharmacol. **40:** 149–155, 1991.

GHAI, G., ZIMMERMAN, M. B., AND HOPKINS, M. F.: Evidence for A_1 and A_2 adenosine receptors in guinea pig trachea. Life Sci. **41:** 1215–1224, 1987.

GHARIB, A., DELTON, I., LAGARDE, M., AND SARDA, N.: Evidence for adenosine A_{2b} receptor in the rat pineal gland. Eur. J. Pharmacol. **225:** 359–360, 1992.

GIROS, B., SOKOLOFF, P., MARTRES, M. P., RIOU, J. F., EMORINE, L. J., AND SCHWARTZ, J. C.: Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. Nature (Lond.) **342:** 923–926, 1989.

GREGORY, C. Y., ABRAMS, T. A., AND HALL, M. O.: Stimulation of A_2 adenosine receptors inhibits the ingestion of photoreceptor outer segments by retinal pigment epithelium. Invest. Ophthalmol. Visual Sci. **35:** 819–825, 1994.

HANCOCK, D. L., AND COUPAR, I. M.: Functional characterization of the adenosine receptor mediating inhibition of intestinal secretion. Br. J. Pharmacol. **114:** 152–156, 1995.

HARGREAVES, M. B., STOGGALL, S. M., AND COLLIS, M. G.: Evidence that the adenosine receptor mediating relaxation in dog lateral saphenous vein and guinea pig aorta is of the A_{2B} subtype. (Abstract) Br. J. Pharmacol. **102:** 198P, 1991.

HAYNES, J. J., OBIKAO, B., THOMPSON, W. J., AND DOWNEY, J.: Adenosine-induced vasodilation: receptor characterization in pulmonary circulation. Am. J. Physiol. **268:** H1862–H1868, 1995.

HEADRICK, J. P., AND BERNE, R. M.: Endothelium-dependent and -independent relaxations to adenosine in guinea pig aorta. Am. J. Physiol. **259:** H62–H67, 1990.

HIDE, I., PADGETT, W. L., JACOBSON, K. A., AND DALY, J. W.: A_2 adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: characterization with radioligand binding and by activation of adenylate cyclase. Mol. Pharmacol. **41:** 352–359, 1992.

HOLLINGSWORTH, E. B., SEARS, E. B., AND DALY, J. W.: An activator of protein kinase C (phorbol-12-myristate-13-acetate) augments 2-chloradenosine-elicited accumulation of cyclic AMP in guinea pig cerebral cortical particulate preparations. FEBS Lett. **184:** 339–342, 1985.

HÖSLI, E., AND HÖSLI, L.: Autoradiographic studies on the uptake of adenosine and on binding of adenosine analogues in neurons and astrocytes of cultured rat cerebellum and spinal cord. Neuroscience **24:** 621–628, 1988.

HOURANI, S. M., NICHOLLS, J., LEE, B. S., HALFHIDE, E. J., AND KITCHEN, I.: Characterization and ontogeny of P1-purinoceptors on rat vas deferens. Br. J. Pharmacol. **108:** 754–758, 1993.

IMOTO, Y., YATANI, A., REEVES, J. P., CODINA, J., BIRNBAUMER, L., AND BROWN, A. M.: α-subunit of G_s directly activates cardiac calcium channels in lipid bilayers. Am. J. Physiol. **255:** H722–H728, 1988.

IWAMOTO, T., UMEMURA, S., TOYA, Y., UCHIBORI, T., KOGI, K., TAKAGI, N., AND ISHII, M.: Identification of adenosine A_2 receptor-cAMP system in human aortic endothelial cells. Biochem. Biophys. Res. Commun. **199:** 905–910, 1994.

JACOBSON, K. A.: Molecular diversity in the search for selective ligands for A_3-adenosine and ATP receptors. (Abstract) Drug Dev. Res. **37:** 112, 1996.

JACOBSON, K. A., AND SUZUKI, F.: Recent developments in selective agonists and antagonists acting at purine and pyrimidine receptors. Drug Dev. Res. **39:** 289–300, 1996.

JACOBSON, K. A., VAN GALEN, P. J. M., AND WILLIAMS, M.: Perspective: adenosine receptors—pharmacology, structure-activity relationships and therapeutic potential. J. Med. Chem. **35:** 407–422, 1992.

JACOBSON, M. A., CHAKRAVARTY, P. K., JOHNSON, R. J., AND NORTON, R.: Novel selective non-xanthine A_3 adenosine receptor antagonists. (Abstract) Drug Dev. Res. **37:** 131, 1996.

JACOBSON, M. A., JOHNSON, R. G., LUNEAU, C. J., AND SALVATORE, C. A.: Cloning and chromosomal localization of the human A_{2b} adenosine receptor gene (ADORA2B) and its pseudogene. Genomics **27:** 374–376, 1995.

JARVIS, M. F., SCHULZ, R., HUTCHINSON, A. J., DO, U. H., SILLIS, M. A., AND WILLIAMS, M.: [^{3}H]CGS 21680, a selective A_2 adenosine receptor agonist directly labels A2 receptors in rat brain. J. Pharmacol. Exp. Ther. **251:** 888–893, 1989.

JIANG, J. L., VAN RHEE, A. M., MELMAN, N., JI, X. D., AND JACOBSON, K. A.: 6-Phenyl-1,4-dihydropyridine derivatives as potent and selective A_3 adenosine receptor antagonists. J. Med. Chem. **39:** 4667–4675, 1996.

KARTON, Y., JIANG, J. L., JI, X. D., MELMAN, N., OLAH, M. E., STILES, G. L., AND JACOBSON, K. A.: Synthesis and biological activities of flavanoid derivatives as A_3 adenosine receptor antagonists. J. Med. Chem. **39:** 2293–2301, 1996.

KENAKIN, T. P., BOND, R. A., AND BONNER, T. I.: Definition of pharmacological receptors. Pharmacol. Rev. **44:** 351–361, 1992.

KESSEY, K., TROMMER, B. L., OVERSTREET, L. S., JI, T., AND MOGUL, D. J.: A role for adenosine A_2 receptors in the induction of long-term potentiation in the CA1 region of rat hippocampus. Brain Res. **756:** 184–190, 1997.

KILIC, S., SALIH, M., ANAFARTA, K., BALTACI, S., AND KOSAR, A.: Adenosine: a new agent in the diagnosis of impotence. Int. J. Impot. Res. **6:** 191–198, 1994.

KLOTZ, K.-N., VOGT, H., AND TAWFIC-SCHLIEPER, H.: Comparison of A_1 adenosine receptors in brain from different species by radioligand binding and photoaffinity labeling. Naunyn-Schmiedeberg's Arch. Pharmacol. **343:** 196–201, 1991.

KONTNY, E., KVANTA, A., AND FREDHOLM, B. B.: Activation of protein kinase C and elevation of cAMP interact synergistically to raise c-Fos, c-June and AP-1 activity in Jurkat cells. Eur. J. Pharmacol. **227:** 333–338, 1992.

KVANTA, A., AND FREDHOLM, B. B.: Synergistic effects between protein kinase C and cAMP on activator protein-1 activity and differentiation of PC-12 pheochromocytoma cells. J. Mol. Neurosci. **4:** 205–214, 1994.

KVANTA, A., GERWINS, P., JONDAL, M., AND FREDHOLM, B. B.: Stimulation of T-cells OKT3 antibodies increases forskolin binding and cyclic AMP accumulation. Cell Signalling **2:** 461–470, 1990.

KVANTA, A., KONTNY, E., JONDAL, M., OKRET, S., AND FREDHOLM, B. B.: Mitogen stimulation of T-cells increases c-Fos and c-Jun protein levels, AP-1 binding and AP-1 transcriptional activity. Cell Signalling **4:** 275–286, 1992.

KVANTA, A., NORSTEDT, C., JONDAL, M., AND FREDHOLM, B. B.: Activation of protein kinase C via the T-receptors potentiates cyclic AMP responses in T-cells. Naunyn-Schmiedeberg's Arch. Pharmacol. **340:** 715–717, 1989.

LEDENT, C., VAUGEOIS, J.-M., SCHIFFMANN, M. E., CANDERHAEGHEN, J.-J., COSTENTIN, J., HEATH, J. K., VASSART, G., AND PARMENTIER, M.: Aggressiveness, hypoalgesia and high blood pressure in mice lacking adenosine A_{2a} receptor. Nature (Lond.) **388:** 674–678, 1997.

LEVENS, N., BEIL, M., AND SCHULZ, R.: Intrarenal actions of the new adenosine agonist CGS 21680A, selective for the A_2 receptor. J. Pharmacol. Exp. Ther. **257:** 1013–1019, 1991.

LIANG, B. T., AND HALTIWANGER, B.: Adenosine A2a and A2b receptors in cultured fetal chick heart cells: high- and low-affinity coupling to stimulation of myocyte contractility and cAMP accumulation. Circ. Res. **76:** 242–251, 1995.

LIANG, B. T., AND MORLEY, J. F.: A new cyclic AMP-independent, G_s-mediated stimulatory mechanism via the adenosine A_{2a} receptor in the intact cardiac cell. J. Biol. Chem. **271:** 18678–18685, 1996.

LIBERT, F., SCHIFFMANN, S. N., LEFORT, A., PARMENTIER, M., GERARD, C., DUMONT, J. E., VANDERHAEGHEN, J. J., AND VASSART, G.: The orphan receptors cDNA RDC7 encodes an A_1 adenosine receptor. EMBO J. **10:** 1677–1682, 1991.

LINDEN, J.: Structure and function of A_1 adenosine receptors. FASEB J. **5:** 2668–2676, 1991.

LINDEN, J.: Cloned adenosine A_3 receptors: pharmacological properties, species differences and receptor functions. Trends Pharmacol. Sci. **15:** 298–306, 1994.

LINDEN, J., TAYLOR, H. E., ROBEVA, A. S., TUCKER, A. L., STEHLE, J. H., RIVKEES, S. A., FINK, J. S., AND REPPERT, S. M.: Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. Mol. Pharmacol. **44:** 524–532, 1993.

LINDSTRÖM, K., ONGINI, E., AND FREDHOLM, B. B.: The selective adenosine A_{2A} receptor antagonist SCH 58261 discriminates between two different binding sites for [^{3}H]-CGS 21680 in the rat brain. Naunyn-Schmiedeberg's Arch. Pharmacol. **354:** 539–541, 1996.

LONDOS, C., COOPER, D. M. F., AND WOLFF, J.: Subclasses of external adenosine receptors. Proc. Natl. Acad. Sci. USA **77:** 2551–2554, 1980.

LUPICA, C. R., CASS, W. A., ZAHNISER, N. R., AND DUNWIDDIE, T. V.: Effects of the selective adenosine A_2 receptor agonist CGS21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. J. Pharmacol. Exp. Ther. **252:** 1134–1141, 1990.

MADARA, J. L., PATAPOFF, T. W., GILLECE-CASTRO, B., COLGAN, S. P., PARKOS, C. A., DELP, C., AND MRSNY, R. J.: 5′-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. J. Clin. Invest. **91:** 2320–2325, 1993.

MAEKAWA, K., SAITO, D., OBAYASHI, N., UCHIDA, S., AND HARAOKA, S.: Role of endothelium-derived nitric oxide and adenosine in functional myocardial hyperemia. Am. J. Physiol. **267:** H166–H173, 1994.

MAENHAUT, C., VAN SANDE, J., LIBERT, F., ABRAMOWITZ, M., PARMENTIER, M., VANDERHARGEN, J. J., DUMONT, J. E., VASSART, G., AND SCHIFFMANN, S.: RDC8 codes for an adenosine A_2 receptor with physiological constitutive activity. Biochem. Biophys. Res. Commun. **173:** 1169–1178, 1990.

MANN, J. S., AND HOLGATE, S. T.: Specific antagonism of adenosine-induced bronchoconstriction is asthma by oral theophylline. Br. J. Clin. Pharmacol. **19:** 685–692, 1985.

MARQUARDT, D. L., PARKER, C. W., AND SULLIVAN, T. J.: Potentiation of mast cell mediator release by adenosine. J. Immunol. **120:** 871–878, 1978.

MARQUARDT, D. L., AND WALKER, L. L.: Modulation of mast cell responses to adenosine by agents that alter protein kinase C activity. Biochem. Pharmacol. **39:** 1929–1934, 1990.

MARQUARDT, D. L., AND WALKER, L. L.: Inhibition of protein kinase A fails to alter mast cell adenosine responsiveness. Agents Actions **43:** 7–12, 1994.

MARQUARDT, D. L., WALKER, L. L., AND HEINEMANN, S.: Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. J. Immunol. **152:** 4508–4515, 1994.

MARTIN, P. L.: Relative agonist potencies of C2-substituted analogues of adenosine: evidence of adenosine A_{2b} receptors in the guinea pig aorta. Eur. J. Pharmacol. **216:** 235–242, 1992.

MARTIN, P. L., AND POTTS, A. A.: The endothelium of the rat renal artery plays an obligatory role in A_2 adenosine receptor-mediated relaxation induced by 5′-N- ethylcarboxamidoadenosine and N^6-cyclopentyladenosine. J. Pharmacol. Exp. Ther. **270:** 893–899, 1994.

MARTIN, P. L., UEEDA, M., AND OLSSON, R. A.: 2-Phenylethoxy-9-methyladenine: an adenosine receptor antagonist that discriminates between A_2 adenosine receptors in the aorta and the coronary vessels from the guinea pig. J. Pharmacol. Exp. Ther. **265:** 248–253, 1993.

MARTINSON, E. A., JOHNSON, R. A., AND WELLS, J. N.: Potent adenosine receptor antagonists that are selective for the A_1 receptor subtype. Mol. Pharmacol. **31:** 247–252, 1987.

MATEO, J., CASTRO, E., ZWILLER, J., AUNIS, D., AND MIRAS-PORTUGAL, M. T.: 5′-(n-ethylcarboxamido)adenosine inhibits Ca^{2+} influx and activates a protein phosphatase in bovine adrenal chromaffin cells. J. Neurochem. **64:** 77–84, 1995.

MILLIGAN, G., MARSHALL, F., AND REES, S.: G_{16} as a universal G protein adapter: implications for agonist screening strategies. Trends Pharmacol. Sci. **17:** 235–237, 1996.

MOGUL, D. J., ADAMS, M. E., AND FOX, A. P.: Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca^{2+} current in hippocampal CA3 neurons. Neuron **10:** 327–334, 1993.

MOSER, G. H., SCHRADER, J., AND DEUSSEN, A.: Turnover of adenosine in plasma of human and dog blood. Am. J. Physiol. **256:** C799–C806, 1989.

MOSQUEDA-GARCIA, R., APPALSAMY, M., AND ROBERTSON, D.: Mechanisms of the central cardiovascular effects of adenosine: interaction with brain L-glutamate. (Abstract) Neuroscience **15:** 1178, 1989.

MOSQUEDA-GARCIA, R., TSENG, C. J., APPALSAMY, M., BECK, C., AND ROBERTSON, D.: Cardiovascular excitatory effects of adenosine in the nucleus of the solitary tract. Hypertension **18:** 494–502, 1991.

MULLANE, K.: Acadesine: the prototype adenosine regulating agent for reducing myocardial injury. Cardiovasc. Res. **27:** 43–47, 1993.

MUNDELL, S. J., BENOVIC, J. L., AND KELLY, E.: A dominant negative mutant of G protein-coupled receptor kinase 2 selectively attenuates adenosine A_2 receptor desensitization. Mol. Pharmacol. **51:** 991–998, 1997.

MURRISON, E. M., GOODSON, S. J., EDBROOKE, M. R., AND HARRIS, C. A.: Cloning and characterization of the human adenosine A_3 receptor gene. FEBS Lett. **384:** 243–246, 1996.

MURTHY, K. S., MCHENRY, L., GRIDER, J. R., AND MAKHLOUF, G. M.: Adenosine A_1 and A_{2b} receptors coupled to distinct interactive signaling pathways in intestinal muscle cells. J. Pharmacol. Exp. Ther. **274:** 300–306, 1995.

NAKANE, T., AND CHIBA, S.: Adenosine constricts the isolated and perfused monkey coronary artery. Heart Vessels **5:** 71–75, 1990.

NANOFF, C., JACOBSON, K. A., AND STILES, G. L.: The A_2 adenosine receptor: guanine nucleotide modulation of agonist binding is enhanced by proteolysis. Mol. Pharmacol. **39:** 130–135, 1991.

NEES, S., HERZOG, V., BECKER, B. F., BOCK, M., DES ROSIERS, C., AND GERLACH, E.: The coronary endothelium: a highly active metabolic barrier for adenosine. Basic Res. Cardiol. **80:** 515–529, 1985.

NEGLISHI, M., SUGIMOTO, Y., AND ICHIKAWA, A.: Molecular mechanisms of diverse actions of prostanoid receptors. Biochim. Biophys. Acta **1259:** 109–120, 1995.

NGUYEN, T., SUNAHARA, R., MARCHESE, A., VAN TOL, H. H. M., SEEMAN, P., AND O'DOWD, B. F.: Transcription of a human dopamine D_5 pseudogene. Biochem. Biophys. Res. Commun. **181:** 16–21, 1991.

NICHOLLS, J., BROWNHILL, V. R., AND HOURANI, S. M. O.: Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle muscularis mucosae. Br. J. Pharmacol. **117:** 170–174, 1996.

NORSTEDT, C., AND FREDHOLM, B. B.: Phorbol-12,13-dibutirate enhances the cyclic AMP accumulation in rat hippocampal slices induced by adenosine analogues. Naunyn-Schmiedeberg's Arch. Pharmacol. **335:** 136–142, 1987.

NORSTEDT, C., KVANTA, A., VAN DER PLOEG, I., AND FREDHOLM, B. B.: Dual effects of protein kinase C on receptor-stimulated cAMP accumulation in a human T-cell leukemia line. Eur. J. Pharmacol. **172:** 51–62, 1989.

NYCE, J. W., AND METZGER, W. J.: DNA antisense therapy for asthma in an animal model. Nature (Lond.) **97:** 721–725, 1997.

OFFERMANNS, S., AND SIMON, M. I.: $G\alpha_{15}$ and $G\alpha_{16}$ couple a wide variety of receptors to phospholipase C. J. Biol. Chem. **270:** 15175–15180, 1995.

OKADA, M., MIZUNO, K., AND KANEKO, S.: Adenosine A_1 and A_2 receptors modualte extracellular dopamine levels in rat striatum. Neurosci. Lett. **212:** 53–56, 1996.

OLAH, M. E.: Identification of A_{2a} adenosine receptor domain involved in selective coupling to G_s: analysis of chimeric A_1/A_{2a} adenosine receptors. J. Biol. Chem. **272:** 337–344, 1997.

OLSSON, R. A.: Adenosine receptors in the cardiovascular system. Drug Dev. Res. **39:** 301–307, 1996.

OLSSON, R. A., DAVIS, C. C., AND KHOURI, E. M.: Coronary vasoactivity of adenosine covalently linked to polylysine. Life Sci. **21:** 1343–1350, 1977.

OLSSON, R. A., AND PEARSON, J. D.: Cardiovascular purinoceptors. Pharmacol. Rev. **70:** 761–845, 1990.

ONGINI, E., DIONISOTTI, S., MORELLI, M., FERRE, S., SVENNINGSSON, P., FUXE, K., AND FREDHOLM, B. B.: Neuropharmacology of the adenosine A_{2A} receptors. Drug Dev. Res. **39:** 450–460, 1996.

ONGINI, E., AND FREDHOLM, B. B.: Pharmacology of adenosine A_{2A} receptors. Trends Pharmacol. Sci. **17:** 364–372, 1996.

PALMER, T. M., JACOBSON, K. A., AND STILES, G. L.: Immunological identification of A_2 adenosine receptors by two antipeptide antibody preparations. Mol. Pharmacol. **42:** 391–397, 1992.

PALMER, T. M., AND STILES, G. L.: Adenosine receptors. Neuropharmacology **34:** 683–694, 1995.

PALMER, T. M., AND STILES, G. L.: Identification of an A_{2a} adenosine receptor domain specifically responsible for mediating of short-term desensitization. Biochemistry **36:** 832–838, 1997.

PATON, D. M.: Structure-activity relations for presynaptic inhibition of noradrenergic and cholinergic transmission by adenosine: evidence for action on A_1 receptors. J. Auton. Pharmacol. **1:** 287–290, 1981.

PEACHELL, P. T., LICHTENSTEIN, L. M., AND SCHLEIMER, R. P.: Differential regulation of human basophil and lung mast cell function by adenosine. J. Pharmacol. Exp. Ther. **256:** 717–726, 1991.

PEAKMAN, M. C., AND HILL, S. J.: Adenosine A_{2b}-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. Br. J. Pharmacol. **111:** 191–198, 1994.

PEAKMAN, M. C., AND HILL, S. J.: Adenosine A_1 receptor-mediated inhibition of cyclic AMP accumulation in type-2 but not type-1 rat astrocytes. Eur. J. Pharmacol. **306:** 281–289, 1996.

PETERFREUND, R. A., MACCOLLIN, M., GUSELLA, J., AND FINK, J. S.: Structure of the human A_{2a} adenosine receptor gene. (Abstract) Drug Dev. Res. **31:** 306, 1994.

PHILLIPS, G. D., NG, W. H., CHURCH, M. K., AND HOLGATE, S. T.: The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5′-monophosphate, and allergen in atopic nonasthmatic subjects. Am. Rev. Respir. Dis. **90:** 9–13, 1990.

PHILLIPS, G. D., RAFFERTY, P., BEASLEY, R., AND HOLGATE, S. T.: Effect of oral terfenadine on the bronchoconstrictor response to inhaled histamine and adenosine 5′-monophosphate in non-atopic asthma. Thorax **87:** 939–945, 1987.

PHILLIPS, G. D., SCOTT, V. L., RICHARDS, R., AND HOLGATE, S. T.: Effect of nedrocromil sodium and sodium cromoglycate against bronchoconstriction induced by inhaled adenosine 5′-monophosphate. Eur. Respir. J. **2:** 210–217, 1989.

PHILLIS, J. W., O'REGAN, M. H., AND PERKINS, L. M.: Effect of adenosine receptor agonist on spontaneous and K^+-evoked acetylcholine release from the in vivo rat cerebral cortex. Brain Res. **605:** 293–297, 1993a.

PHILLIS, J. W., PERKINS, L. M., AND O'REGAN, M. H.: Potassium-evoked efflux of transmitter amino acids and purines from rat cerebral cortex. Brain Res. Bull. **31:** 547–552, 1993b.

PIERCE, K. D., FURLONG, T. J., SELBIE, L. A., AND SHINE, J.: Molecular cloning and expression of an adenosine A_{2b} receptor from human brain. Biochem. Biophys. Res. Commun. **187:** 86–93, 1992.

PIERSEN, C. E., TRUE, C. D., AND WELLS, J. N.: A carboxyl-terminally truncated mutant and nonglycosylated A_{2a} adenosine receptors retain ligand binding. Mol. Pharmacol. **45:** 861–870, 1994.

POLOSA, R., NG, W. H., CRIMI, N., VANCERI, C., HOLGATE, S. T., CHURCH, M. K., AND MISTRETTA, A.: Release of mast cell-derived mediators after endobronchial adenosine challenge in asthma. Am. J. Respir. Crit. Care Med. **151:** 624–629, 1995.

PORZIG, H., GUTKNECHT, R., KOSTOVA, G., AND THALMEIER, K.: G protein-coupled receptors in normal human erytroid progenitor cells. Naunyn-Schmiedeberg's Arch. Pharmacol. **353:** 11–20, 1995.

POUCHER, S. M., KEDDIE, J. R., SINGH, P., STOGGALL, S. M., CAULKETT, P. W. R., JONES, G., AND COLLIS, M. G.: The in vitro pharmacology of ZM 241385, a potent non-xanthine, A_{2a} selective adenosine receptor antagonist. Br. J. Pharmacol. **115:** 1096–1102, 1995.

PRENTICE, D. J., AND HOURANI, S. M. O.: Activation of multiple sites by adenosine analogues in the rat isolated aorta. Br. J. Pharmacol. **118:** 1509–1517, 1996.

PRENTICE, D. J., AND HOURANI, S. M. O.: Adenosine analogues relax guinea-pig taenia caeci via an adenosine A_{2B} receptor and a xanthine-resistant site. Eur. J. Pharmacol. **323:** 103–106, 1997.

PUFFINBARGER, N. K., HANSEN, K. R., RESTA, R., LAURENT, A. B., KNUDSEN, T. B., MADARA, J. L., AND THOMPSON, L. F.: Production and characterization of multiple antigenic peptide antibodies to the adenosine A_{2b} receptor. Mol. Pharmacol. **47:** 1126–1132, 1995.

RAFFERTY, P., BEASLEY, C. R., AND HOLGATE, S. T.: The contribution of histamine to immediate bronchoconstriction provoked by inhaled allergen and adenosine-5′monophosphate in atopic asthma. Am. Rev. Respir. Dis. **136:** 369–373, 1987.

RAMKUMAR, V., STILES, G. L., BEAVEN, M. A., AND ALI, H.: The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. J. Biol. Chem. **268:** 16887–16890, 1993.

RAMKUMAR, V., WILSON, M., DHANRAJ, D. N., GETTYS, T. W., AND ALI, H.: Dexamethasone up-regulates A_3 adenosine receptors in rat basophilic leukemia (RBL-2HE) cells. J. Immunol. **154:** 5437–5443, 1995.

REN, H., AND STILES, G. L.: Characterization of the human A_1 adenosine receptor gene: evidence for alternative splicing. J. Biol. Chem. **269:** 3104–3110, 1994.

RIVKEES, S. A., AND REPPERT, S. M.: RFL9 encodes an A_{2b}-adenosine receptor. Mol. Endocrinol. **6:** 1598–1604, 1992.

ROBEVA, A. S., WOODARD, R. L., JIN, X., GAO, Z., BHATTACHARYA, S., TAYLOR, H. E., ROSIN, D. L., AND LINDEN, J.: Molecular characterization of recombinant human adenosine receptors. Drug Dev. Res. **39:** 243–252, 1996.

ROMANO, F. D., MACDONALD, S. G., AND DOBSON, J. G., JR.: Adenosine receptor coupling to adenylate cyclase of rat ventricular myocyte membrane. Am. J. Physiol. **257:** H1088–H1095, 1989.

RUBANYI, G., AND VANHOUTTE, P. M.: Endothelium removal decreases relaxations of canine coronary arteries caused by β-adrenergic agonists and adenosine. J. Cardiovasc. Pharmacol. **7:** 139–144, 1985.

RUBINO, A., RALEVIC, V., AND BURNSTOCK, G.: Contribution of P1-(A_{2b} subtype) and P2-purinoceptors to the control of vascular tone in the rate isolate mesenteric arterial bed. Br. J. Pharmacol. **115:** 648–652, 1995.

SALVATORE, C. A., JACOBSON, M. A., TAYLOR, H. E., LINDEN, J., AND JOHNSON, R. G.: Molecular cloning and characterization of the human A_3 adenosine receptor. Proc. Natl. Acad. Sci. USA **90:** 10365–10369, 1993.

SATTIN, A., AND RALL, T. W.: The effect of adenosine and adenine nucleotides on the adenosine 3′,5′-phosphate content of guinea pig cerebral cortex slices. Mol. Pharmacol. **6:** 13–23, 1970.

SCAMPS, F., RYBIN, V., PUCEAT, M., TKACHUK, V., AND VASSORT, G.: A G_s protein couples P2-purinergic stimulation to cardiac Ca channels without cyclic AMP production. J. Gen. Physiol. **100:** 675–701, 1992.

SCHWARTZ, L. B.: Tryptase, a mediator of human mast cells. J. Allergy Clin. Immunol. **86:** 594–598, 1990.

SEBASTIÃO, A. M., AND RIBEIRO, J. A.: Adenosine A_2 receptor-mediated excitatory actions on the nervous system. Prog. Neurobiol. **48:** 167–189, 1996.

SOLLEVI, A., LAGERKRANSER, M., IRESTEDT, L., GORDON, E., AND LINDQUIST, C.: Controlled hypotension with adenosine in cerebral aneurysm surgery. Anesthesiology **61:** 400–405, 1984.

STEHLE, J. H., RIVKEES, S. A., LEE, J. J., WEAVER, D. R., DEEDS, J. D., AND REPPERT, S. M.: Molecular cloning and expression of the cDNA for a novel A_2- adenosine receptor subtype. Mol. Endocrinol. **6:** 384–393, 1992.

STEIN, B., MENDE, U., NEUMANN, J., SCHMITZ, W., AND SCHOLZ, H.: Pertussis toxin unmasks stimulatory myocardial A_2-adenosine receptors on ventricular cardiomyocytes. J. Mol. Cell. Cardiol. **25:** 655–659, 1993.

STRICKLER, J., JACOBSON, K. A., AND LIANG, B. T.: Direct preconditioning of cultured chick ventricular myocytes: novel functions of cardiac adenosine A_{2a} and A_3 receptors. J. Clin. Invest. **98:** 1773–1779, 1996.

STROHMEIER, G. R., REPPERT, S. M., LENCER, W. I., AND MADARA, J. L.: The A_{2b} adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. J. Biol. Chem. **270:** 2387–2394, 1995.

SVENNINGSSON, P., AND FREDHOLM, B. B.: Glucocorticoids regulate the expression of adenosine A_1 but not A_{2A} receptors in rat brain. J. Pharmacol. Exp. Ther. **280:** 1094–1101, 1997.

TAMAOKI, J., TAGAYA, E., CHIYOTANI, A., TAKEMURA, H., NAGAI, A., KONNO, K., ONUKI, T., AND NITTA, S.: Effect of adenosine on adrenergic neurotransmission and modulation by endothelium in canine pulmonary artery. Am. J. Physiol. **272:** H1100–H1105, 1997.

TOFOVIC, S. P., BRANCH, K. R., OLIVER, R. D., MAGEE, W. D., AND JACKSON, E. K.: Caffeine potentiates vasodilator-induced renin release. J. Pharmacol. Exp. Ther. **256:** 850–860, 1991.

TOWNSEND-NICHOLSON, A., BAKER, E., SUTHERLAND, G. R., AND SCHOFIELD, P. R.: Localization of the adenosine A_{2b} receptor subtype gene (ADORA2B) to chromosome 17p11.2-p12 by FISH and PCR screening of somatic cell hybrids. Genomics **25:** 605–607, 1995.

TSENG, C. J., BIAGGIONI, I., APPALSAMY, M., AND ROBERTSON, D.: Purinergic receptors in the brainstem mediate hypotension and bradycardia. Hypertension **11:** 191–197, 1988.

TUCKER, A., HOLETON, D., CHO, E. S., TAYLOR, A., AND LINDEN, J.: Chimeric canine A_1/A_{2A} adenosine receptors identify intracellular domains important for determining selective G protein coupling. (Abstract) Drug Dev. Res. **37:** 110, 1996.

TUCKER, A. L., AND LINDEN, J.: Cloned receptors and cardiovascular responses to adenosine. Cardiovasc. Res. **27:** 62–67, 1993.

TURNER, J. T., CAMDEN, J. M., KANSRA, S., SHELTON-JAMES, D., WU, H., AND HALENDA, S. P.: Potentiation of cyclic adenosine monophosphate production by thrombin in the human erythroleukemia cell line HEL. J. Pharmacol. Exp. Ther. **263:** 708–716, 1992.

UKENA, D., JACOBSON, K. A., KIRK, K. L., AND DALY, J. W.: A [^{3}H]amine congener of 1,3-dipropyl-8-phenylxanthine: a new radioligand for A_2 adenosine receptors of human platelets. FEBS Lett. **199:** 269–274, 1986.

UKENA, D., SCHIRREN, C. G., AND SCHWABE, U.: Effects of enprofylline on A_1 and A_2 adenosine receptors. Eur. J. Pharmacol. **117:** 25–33, 1985.

VAN CALKER, D., MUELLER, M., AND HAMPRECHT, B.: Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J. Neurochem. **33:** 999–1005, 1979.

VAN DER PLOEG, I., AHLBERG, S., PARKINSON, F. E., OLSSON, R. A., AND FREDHOLM, B. B.: Functional characterization of adenosine A2 receptors in Jurkat cells and PC12 cells using adenosine receptor agonists. Naunyn-Schmiedeberg's Arch. Pharmacol. **353:** 250–260, 1996.

VAN GALEN, P. J. M., VAN BERGEN, A. H., GALLO-RODRIGUEZ, C., MELMAN, N., OLAH, M. E., IJZERMAN, A. P., STILES, G. L., AND JACOBSON, K. A.: A binding site model and structure-activity relationships for rat A_3 adenosine receptor. Mol. Pharmacol. **45:** 1101–1111, 1994.

VAN RHEE, A. M., JIANG, J. L., MELMAN, N., OLAH, M. E., STILES, G. L., AND JACOBSON, K. A.: Interaction of 1,4-dihydropyridine and pyridine derivatives with adenosine receptors: selectivity for A_3 receptors. J. Med. Chem. **39:** 2980–2989, 1996.

VERANI, M. S., MAHMARIAN, J. J., HIXSON, J. B., BOYCE, T. M., AND STAUDACHER, R. A.: Diagnosis of coronary artery disease by controlled coronary vasodilatation with adenosine and thallium-201 scintigraphy in patients unable to exercise. Circulation **82:** 80–87, 1990.

VOLLMER, W. M., BUIST, A. S., AND OSBORNE, M. L.: Twenty year trends in hospital discharges for asthma among members of a health maintenance organization. J. Clin. Epidemiol. **45:** 999–1006, 1992.

WAKADE, A. R., AND WAKADE, T. D.: Inhibition of noradrenaline release by adenosine. J. Physiol. **282:** 35–49, 1978.

WALDAY, P., AND AAS, P.: Prejunctional stimulation of cholinergic nerves in rat airway smooth muscle by an adenosine analogue. Pulm. Pharmacol. **4:** 114–119, 1991.

WEBB, R. L., SILLS, M. A., CHOVAN, J. P., BALWIERCZAK, J. L., AND FRANCIS, J. E.: CGS 21680: a potent selective adenosine A_2 receptor agonist. Cardiovasc. Drug Rev. **10:** 26–53, 1992.

WEINBERGER, M., AND HENDELES, L.: Theophylline in asthma. N. Engl. J. Med. **334:** 1380–1388, 1996.

WEISS, K. B., GERGEN, P. J., AND WAGENER, D. K.: Breathing better or wheezing worse? The changing epidemiology of asthma morbidity and mortality. Annu. Rev. Public Health **14:** 491–513, 1993.

WILKIE, T., SCHERLE, P., STRATHMANN, M. P., SLEPAK, V. Z., AND SIMON, M. I.: Characterization of G protein alpha subunits in the G_q class: expression in murine tissues and in stromal and hematopoietic cell lines. Proc. Natl. Acad. Sci. USA **88:** 10049–10053, 1991.

XU, H., STEIN, B., AND LIANG, B. T.: Characterization of a stimulatory adenosine A_{2a} receptor in adult rat ventricular myocyte. Am. J. Physiol. **270:** H1655–H1661, 1996.

YAKEL, J. L., WARREN, R. A., REPPERT, S. M., AND NORTH, R. A.: Functional expression of adenosine A_{2b} receptor in *Xenopus* oocytes. Mol. Pharmacol. **43:** 277–280, 1993.

YEN, M.-H., WU, C.-C., AND CHIOU, W.-F.: Partially endothelium-dependent vasodilator effect of adenosine in rat aorta. Hypertension **11:** 514–518, 1988.

ZHOU, Q.-Y., LI, C., OLAH, M. E., JOHNSON, R. A., STILES, G. L., AND CIVELLI, O.: Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. Proc. Natl. Acad. Sci. USA **89:** 7432–7436, 1992.

ZOCCHI, C., ONGINI, E., CONTI, A., MONOPOLI, A., NEGRETTI, A., BARALDI, P. G., AND DIONISOTTI, S.: The non-xanthine heterocyclic compound SCH 58261 is a new potent and selective A_{2a} adenosine receptor antagonist. J. Pharmacol. Exp. Ther. **276:** 398–404, 1996a.

ZOCCHI, C., ONGINI, E., FERRARA, S., BARALDI, P. G., AND DIONISOTTI, S.: Binding of the radioligand [^{3}H]-SCH 58261, a new non-xanthine A_{2A} adenosine receptor antagonist, to rat striatal membranes. Br. J. Pharmacol. **117:** 1381–1386, 1996b.

Adenosine A_{2B} receptors: a novel therapeutic target in asthma?

Igor Feoktistov, Riccardo Polosa, Stephen T. Holgate and Italo Biaggioni

Adenosine is an endogenous nucleoside that modulates many physiological processes. Its actions are mediated by interaction with specific cell membrane receptors. Four subtypes of adenosine receptor have been cloned: A_1, A_{2A}, A_{2B} and A_3. Significant advancement has been made in our understanding of the molecular pharmacology and physiological relevance of adenosine receptors but our knowledge of A_{2B} receptors lags behind that of other receptor types. Only recently have potentially important functions been discovered for the A_{2B} receptors, prompting a renewed interest in this receptor type. A_{2B} receptors have been implicated in the regulation of vascular smooth muscle tone, cell growth, intestinal function and neurosecretion. In this review, **Igor Feoktistov, Riccardo Polosa, Stephen Holgate and Italo Biaggioni** focus on the role of A_{2B} receptors in mast cell activation and the potential relevance of this action on asthma.

I. Feoktistov, Assistant Professor, Division of Cardiology and **I. Biaggioni,** Associate Professor, Clinical Pharmacology, Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, TN 37232-2195, USA, and **R. Polosa,** Visiting Scholar, and **S. T. Holgate,** Clinical Professor, Division of Immunopharmacology, University of Southampton, Southampton, UK SO16 6YD.

Among the many actions of adenosine[1,2], several lines of evidence suggest a contribution to asthma. When administered by inhalation to patients with asthma but not to normal subjects, adenosine provokes concentration-related bronchoconstriction[3]. In addition, inhalation of its related nucleotides AMP and ADP produces almost identical effects on the airways[4], since both nucleotides are dephosphorylated to yield adenosine.

The ability of the adenosine uptake inhibitor dipyridamole to potentiate adenosine-induced bronchoconstriction indicates that the nucleoside interacts with cell-surface purine receptors[5]. Moreover, theophylline, which also acts as an adenosine receptor antagonist, has been shown to produce a greater protection against adenosine than against histamine-induced bronchoconstriction[6]. One question raised by this observation was the anti-asthmatic activity of the xanthine derivative enprofylline, which had previously been thought to lack adenosine receptor antagonist activity[7]; however, it has recently been shown that this drug does, in fact, possess A_{2B} receptor antagonist properties[8]. Evidence that mediators derived from mast cells are involved in the adenosine response is indicated by studies *in vitro* which have shown that adenosine and its analogues markedly enhance histamine release[9] and prostanoid generation[10] elicited from immunologically stimulated human lung mast cells. These findings support clinical observations that premedication with antihistamines[11,12] and potent inhibitors of cyclooxygenase[13,14] inhibit the acute bronchoconstrictor response to inhaled AMP in asthmatic subjects.

More direct evidence that preformed and newly generated mediators released from airway mast cells can indeed play a role in adenosine-induced responses comes from a study showing a significant rise in plasma levels of histamine occur after AMP challenge[15]. It has also been shown that high concentrations of mast-cell-derived histamine and tryptase occur in the bronchoalveolar lavage (BAL) fluid of asthmatic patients after endobronchial challenge with AMP (Ref. 16). Nasal provocation with AMP indicates that this purine nucleotide can also mimic many of the symptoms of rhinitis and release histamine into the nasal cavity[17,18]. The potent and specific histamine H_1 receptor antagonist cetirizine abolishes nasal symptoms in all subjects studied so far[17].

Activation of neural pathways might also contribute to the contractile airway response to adenosine in asthma[19–22]. Bronchoconstriction induced by 2-chloroadenosine in guinea-pigs *in vivo* might well have a capsaicin-sensitive component[23], indicating that the release of contractile neuropeptides from sensory nerve endings might be of some importance in mediating the airway effects of purine derivatives. In support of this view, repeated exposure of asthmatic airways to AMP results in cross-tachyphylaxis with bradykinin[24].

A putative role for adenosine in airway inflammation

Adenosine is increased under inflammatory conditions of the airways. High concentrations of adenosine have been measured in the BAL fluid of subjects with asthma and chronic bronchitis compared with normal controls[25].

All cells contain adenosine and adenine nucleotides. Adenosine release has been shown to occur from rat peritoneal mast cells upon IgE-dependent challenge[26]. Mast cells might also be a source of adenosine released into airway fluid since the concentration of adenosine tends to be higher in those asthmatic subjects who also have high levels of histamine in the airways[25]. Platelets might be another source of adenosine: platelet activation elicits the release of ADP, which could then be converted to adenosine. Similarly, neutrophils release 5'-AMP, which is rapidly converted to adenosine by a specific ecto-5'-nucleotidase[27].

Once generated, adenosine has the capacity to promote a large variety of effector functions in the airways. Aside from its potential role as a mediator of bronchoconstriction, it might also function as a novel paracrine mediator that contributes to various aspects of the inflammatory response. Adenosine causes plasma exudation

  0165 – 6147/98/$19.00 PII: S0165-6147(98)01179-1

Box 1. Molecular pharmacology of adenosine A_{2B} receptors

It is generally accepted that both adenosine A_{2A} and A_{2B} receptors activate adenylate cyclase via their coupling to G_s proteins in virtually every cell where they are expressed. As well as activating adenylate cyclase, other intracellular signalling pathways have been found to be coupled functionally to A_{2B} receptors (see Fig.).

Recombinant rat A_{2B} receptors expressed in *Xenopus* oocytes activate Ca^{2+}-dependent Cl^- conductance, presumably by stimulating phospholipase C (Ref. 1). Likewise, it has been proposed that A_{2B} receptors stimulate phospholipase C in mouse bone marrow-derived mast cells[2]. Regulatory proteins of the G_q family are thought to play a role in the coupling of A_{2B} receptors to β-phospholipase C in human mast HMC-1 cells[3] and dog mastocytoma cells[4], since this process is unaffected by treatment with pertussis or cholera toxins. A_{2B} receptor-mediated stimulation of β-phospholipase C results in the mobilization of intracellular Ca^{2+} in HMC-1 cells and eventually promotes the synthesis of interleukin 8 (IL-8) (Ref. 3).

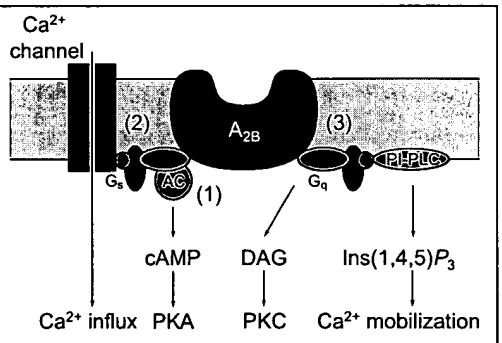


Fig. Diagram showing the intracellular pathways directly coupled to the adenosine A_{2B} receptor in various cell types. (1) A_{2B} receptors are coupled to adenylate cyclase (AC) via G_s protein in virtually every cell studied. Activation of this pathway results in the accumulation of cAMP and stimulation of protein kinase A (PKA). (2) In human erythroleukaemia and Jurkat T cells, A_{2B} receptors also increase Ca^{2+} influx by coupling with G_s protein. (3) In contrast, A_{2B} receptors are coupled to phosphatidylinositol-specific phospholipase C (PI-PLC) via a G protein of the G_q family in mast cells. Activation of this pathway results in an increase in diacylglycerol (DAG) and inositol (1,4,5)trisphosphate [Ins(1,45)P_3]. Diacylglycerol stimulates protein kinase C (PKC). Inositol trisphosphate activates the mobilization of Ca^{2+} from intracellular stores.

The stimulation of A_{2B} receptors also increases the concentration of intracellular Ca^{2+} in human erythroleukaemia cells but via a different mechanism. A_{2B} receptors potentiate Ca^{2+} influx in these cells through a cholera toxin-sensitive mechanism; in contrast with the mobilization of intracellular Ca^{2+} observed in HMC-1 cells. Even though this process is coupled to G_s proteins, it is independent of cAMP. It has been suggested that $G\alpha_s$ can directly stimulate a putative Ca^{2+} channel[5], as demonstrated for other G_s-coupled receptors[6]. A_{2B} receptors are also coupled to a Ca^{2+} channel via a mechanism sensitive to cholera toxin and independent of cAMP in Jurkat T cells[7]. In addition, the activation of A_{2B} receptors results in significant potentiation of P-type but not N-type Ca^{2+} current in pyramidal neurones from the CA3 region of the guinea-pig hippocampus. This mechanism is thought to be mediated by adenylate cyclase because this potentiation can be inhibited by blocking the cAMP-dependent protein kinase[8].

In summary, current evidence suggests that the actions of A_{2B} receptors can be mediated not only by cAMP but also by other intracellular pathways that could vary between cells. It is interesting that, as far as intracellular pathways are concerned, A_{2B} receptors have as much in common with A_1 or A_3 receptors (activation of phospholipase C, modulation of ion channels) as with A_{2A} receptors (activation of adenylate cyclase). The A_{2B}-mediated stimulation of protein kinase C and increase in intracellular Ca^{2+} seems to be more relevant to mechanisms of mast cell activation than increases in cAMP concentrations.

References

1 Yakel, J. L., Warren, R. A., Reppert, S. M. and North, R. A. (1993) *Mol. Pharmacol.* 43, 277–280
2 Marquardt, D. L., Walker, L. L. and Heinemann, S. (1994) *J. Immunol.* 152, 4508–4515
3 Feoktistov, I. and Biaggioni, I. (1995) *J. Clin. Invest.* 96, 1979–1986
4 Auchampach, J. A., Jin, X., Wan, T. C., Caughey, G. H. and Linden, J. (1997) *Mol. Pharmacol.* 52, 846–860
5 Feoktistov, I., Murray, J. J. and Biaggioni, I. (1994) *Mol. Pharmacol.* 45, 1160–1167
6 Imoto, Y., Yatani, A., Reeves, J. P., Codina, J., Birnbaumer, L. and Brown, A. M. (1988) *Am. J. Physiol.* 255, H722–H728
7 Mirabet, M., Mallow, J., Lluis, C. and Franco, R. (1997) *Br. J. Pharmacol.* 122, 1075–1082
8 Mogul, D. J., Adams, M. E. and Fox, A. P. (1993) *Neuron* 10, 327–334

and increased bronchial blood flow[28], the ensuing presence of plasma proteins in the interstitium producing fluid efflux via osmosis and, hence, oedema. Adenosine is also able to promote inflammation by inducing neutrophil chemotaxis[29] and by enhancing histamine release from immunologically activated human lung mast cells[10,30].

Airway responsiveness to adenosine and clinical correlates

Confirmation of the bronchoconstrictor response to adenosine *in vivo* in asthma subjects has been obtained *in vitro*: bronchi from asthmatic subjects are more sensitive to adenosine than those obtained from non-asthmatic controls[31]. In this regard, adenosine could be unique since hyperresponsiveness *in vitro* has not been demonstrated using other mediators such as histamine and leukotriene C_4 (Ref. 31). In humans, the response of the airways to inhaled adenosine is not accompanied by a late phase of bronchoconstriction or an increase in non-specific bronchial responsiveness[32]. Responsiveness of the airways to inhaled adenine derivatives correlates only weakly with more direct indices of airway responsiveness measured with agonists like histamine[4] and methacholine[33].

When compared with non-atopic controls, Phillips *et al.*[15] have shown that atopic subjects are more responsive

to inhaled AMP than they are to methacholine, suggesting that the airway response to these purines might be an index of mast cell priming. The capacity of adenosine to augment mediator release from mast cells in the presence of a 'low-level' second stimulus (which can be immunological or non-immunological) raises the possibility that this nucleoside elicits mediator release in asthmatic human airways by interacting with cytokine-'primed' mast cells on the surface of inflamed airways. Thus, purine-induced bronchoconstriction in asthmatics and atopics might well depend on the state of airway mast cell priming and, indeed, could be useful as a test for this *in vivo*.

Non-smoking adults with chronic obstructive pulmonary disease (COPD) are less responsive to inhaled adenosine than non-smoking asthmatics, whereas the sensitivity to methacholine is similar in both groups[34]. In children, bronchoprovocation tests with inhaled adenosine discriminates asthma from pediatric COPD with a sensitivity and specificity of 85–90%; this is in contrast to methacholine challenge, which fails to distinguish between the two[35]. In this context, adenosine challenge is a useful tool in the diagnosis of asthma when uncertainty exists.

That adenosine responsiveness might prove to be a more appropriate marker of disease activity in relation to asthmatic inflammation than other non-specific stimuli such as histamine or methacholine is supported by a number of clinical studies. For example, inhaled glucocorticosteroids have a significantly greater effect on bronchial hyperresponsiveness to AMP compared with their effects on hyperresponsiveness to direct and neurally acting stimuli[36,37]. Similarly, when a group of allergic asthmatic children were moved to an environment free from house dust mite, a beneficial effect in peak expiratory flow variability occurred, paralleled by a significant improvement in bronchial hyperresponsiveness measured with AMP but not with methacholine[38].

Adenosine receptors in mast cells

The evidence presented so far indicates a unique response to adenosine in asthmatics and suggests that this phenomenon involves mast cell activation. Marquardt *et al.*[39] were the first to report that adenosine, while ineffective alone, potentiates histamine release induced by anti-IgE, concanavalin A, compound 48/80 or the calcium ionophore A23187 in isolated rat mast cells. The mechanisms that mediated the potentiation of these cells was unclear. Stimulation of adenylate cyclase by adenosine can be blocked by the methylxanthine derivative 8-phenyltheophylline but potentiation of histamine release is not, suggesting that these effects are mediated by different adenosine receptors[40].

Because potentiation of rat peritoneal mast cells is insensitive to 8-phenyltheophylline, it was suggested that this effect is mediated by A_3 receptors since the rat A_3 receptor has a remarkably low affinity for xanthines[41]. This possibility was examined in the rat basophilic leukaemia cell line RBL-2H3, which has been used as a model for rat mast cells. It was found that adenosine analogues stimulate phospholipase C, increase the concentration of cytoplasmic Ca^{2+} and potentiate mediator release in these cells with a pharmacological profile consistent with A_3 receptors[42]. Expression of A_3 receptors in RBL-2H3 cells, along with A_{2A} and A_{2B} receptors, has been confirmed by radioligand binding and detection of mRNA[42,43]. Furthermore, A_3 agonists have been reported to provoke bronchoconstriction *in vivo* in BDE strain rats through a process likely involving mast cell activation[44]. Interestingly, A_3 receptors in mast cells have also been implicated in mediating adenosine-induced hypotension in anaesthetized rats[45].

It should be noted that A_1 receptors have also been implicated in adenosine-induced bronchoconstriction in a rabbit model of asthma. An antisense oligodeoxynucleotide targeting A_1 receptors significantly desensitizes rabbits to a challenge with either adenosine or allergen[46], providing substantial evidence for the involvement of A_1 receptors in this model. However, it is unclear to what extent mast cells are involved in this phenomenon or if the effects of adenosine in this model can be explained by a direct effect of A_1 receptors on bronchoconstriction[47].

There is also growing evidence that A_{2B} receptors (see Box 1) modulate mast cell function. Adenosine activates adenylate cyclase and protein kinase C, and potentiates stimulated mediator release in mouse bone marrow-derived mast cells[48]. It appears that the abilities of adenosine to activate protein kinase C, and thereby to augment mast cell degranulation, are independent of changes in the concentration of cAMP (Ref. 49). Both A_{2A} and A_{2B}, but not A_1, transcripts have been detected in mouse bone-marrow-derived mast cells[43]. The failure of the A_{2A}-selective agonist CGS21680 to enhance mediator release suggests that A_{2B} is the receptor type that modulates degranulation of these mast cells[43].

A_{2B} receptors have also been shown to activate the human mast cell line HMC-1 (Ref. 8). HMC-1 cells were derived from a patient with mast cell leukaemia and have a neutral protease content similar to that of human lung mast cells. These cells co-express A_{2A} and A_{2B} receptors, both of which are coupled to adenylate cyclase through G_s proteins. However, only A_{2B} receptors activate HMC-1 cells, as indicated by the stimulation of interleukin 8 (IL-8) secretion using the nonselective A_2 agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) but not with the selective A_{2A} agonist CGS21680. Cyclic AMP does not appear to modulate this process since neither forskolin nor 8-bromo-cAMP influences IL-8 secretion. On the other hand, this effect is mediated by coupling to phospholipase C; this was made evident by the fact that inositol phosphate production increased with consequent mobilization of intracellular Ca^{2+}. These A_{2B} receptor-dependent pathways are stimulated through a G protein that is insensitive to both cholera toxin and pertussis toxin, presumably of the G_q family[8]. The activation of A_{2B} receptors not only stimulates HMC-1 cells directly

but also potentiates phorbol 12-myristate 13-acetate (PMA)-stimulated secretion of IL-8 (Ref. 8). The expression of A_{2B} receptors in HMC-1 cells has recently been confirmed by immunoblotting and fluorescent immunostaining with a specific anti-A_{2B} antibody[50]. A virtually identical mechanism of A_{2B}-mediated activation has been reported in a canine BR mastocytoma cell line. While these cells also express A_1 and A_3 receptors, the A_{2B} receptor seems to be predominant in these cells; this is because adenylate cyclase is stimulated by adenosine agonists and both A_1 and A_3 receptors are negatively coupled to this enzyme through pertussis toxin-sensitive G_i/G_o proteins. Furthermore, adenosine analogues stimulate β-hexaminidase release, inositol phosphate production and intracellular Ca^{2+} mobilization in canine mast cells through a pertussis toxin-insensitive G protein with a pharmacological profile consistent with activation of A_{2B} receptors[51].

Mast cells from different species and even from different anatomical sites within the same species can vary substantially in their morphological and biochemical characteristics. Therefore, it is not surprising that more than one adenosine receptor is involved in the activation of mast cells obtained from different species and tissues. Because of this, it is important to determine the characteristics of adenosine receptors present in human mast cells that are linked to asthma. With regard to A_3 receptors, *in situ* hybridization and reverse transcriptase–polymerase chain reaction techniques have failed to detect A_3 receptors in human parenchymal lung mast cells[52]. In functional studies using parenchymal human lung mast cells obtained from normal sections of excised lung, adenosine analogues do not evoke the release of histamine and leukotriene C_4 directly but potentiate the release of mediators from immunologically activated cells[10]. The order of potency of adenosine analogues suggests that this response is mediated by A_2 receptors. This potentiation was reversed when higher concentrations of adenosine agonists (e.g. 100 μM NECA) were used. In this model, cAMP is known to inhibit histamine release[53], suggesting that cAMP does not mediate the potentiation of mediator release by A_2 receptors. Preliminary studies using double immunostaining with specific anti-A_{2B} and anti-tryptase antibodies demonstrate the presence of A_{2B} receptors in human lung mast cells obtained by bronchoalveolar lavage[50].

Given that inhaled adenosine only affects asthmatic airways and has no effect in controls, there appears to be an intrinsic difference in the way adenosine interacts with mast cells from patients with asthma. The response produced *in vitro* by A_{2B} receptors in HMC-1 cells and in canine BR mastocytoma cells appears to mimic the responses *in vivo* to inhaled adenosine in asthmatics, in that adenosine alone provokes mast cell activation in these cell lines as it does in asthmatics. On the other hand, the response produced *in vitro* by adenosine in mast cells from normal human lung tissue resembles the effect of A_{2B} receptors in mouse bone marrow-derived mast cells, since in both cases adenosine potentiates mast cell activation but does not evoke direct activation. The molecular mechanisms behind these differential A_{2B}-mediated responses in asthmatic compared with normal mast cells, and in HMC-1 cells compared with mouse bone marrow-derived mast cells, remain to be elucidated. Several mechanisms could explain these differences, including diversity at the receptor level, differential coupling of A_{2B} receptors to intracellular pathways, or involvement of other processes that potentiate the activation of A_{2B} receptors in asthmatic mast cells.

A_{2B} receptors as therapeutic targets

Theophylline is an effective drug for the treatment of asthma but it is far from being ideal. For maximum efficacy it is required at the substantial plasma concentration of 20–80 μmol l^{-1} and it has many side-effects, which can be attributed to its nonspecificity. From the studies presented above, it is possible that the blockade of A_{2B} receptors in mast cells might be one of the mechanisms that contribute to the therapeutic efficacy of theophylline in asthma. If this hypothesis proves to be correct, A_{2B} receptors could become therapeutic targets in the development of novel anti-asthma agents. In general, although A_{2B} receptors are recognized to have a lower affinity for agonists compared with other receptor subtypes, this is not true for antagonists. The structure–activity relationship of A_{2B} receptors for adenosine antagonists has not been completely characterized but at least some xanthines are as potent antagonists at A_{2B} receptors as they are at other adenosine receptors[54,55]. Enprofylline, an anti-asthmatic drug, is the most selective A_{2B} receptor antagonist known to date. In early studies, enprofylline was found to be about 20 times more potent in blocking A_2 receptors in the rat hippocampus than A_1 receptors in rat fat cells[56]. Therefore, it was initially proposed that enprofylline could selectively block a subtype of A_2 receptors in the hippocampus[56], now known to correspond to A_{2B} receptors[57]. However, enprofylline was found to be a poor antagonist of A_2 receptors in platelets[58]. Although these findings led to the conclusion that enprofylline was not an adenosine receptor antagonist[58], they can now be explained by the fact that platelets express mainly A_{2A} receptors[54,59].

The belief that enprofylline was not an adenosine receptor antagonist was used as an argument against the hypothesis that adenosine played a role in asthma, since enprofylline proved more effective than theophylline in the treatment of asthma but was thought to act independently of adenosine antagonism[60]. Enprofylline also has a low affinity for A_3 receptors[61]. Recently, it has been shown that enprofylline is equipotent to theophylline as an A_{2B} receptor antagonist of NECA-induced activation of adenylate cyclase in human erythroleukaemia cells (HEL), with a dissociation constant (K_B) of the antagonist–receptor complex of 7 μM (Ref. 8); this is in close agreement with therapeutic plasma levels of enprofylline (5–25 μmol l^{-1}) and earlier results in the

hippocampus[56]. An identical K_i for enprofylline for A_{2B} receptors and a lower affinity of this drug for other receptor subtypes has been confirmed in radioligand binding studies on all four types of human adenosine receptor expressed in CHO cells[62]. Enprofylline is an effective antagonist of A_{2B} receptors in human HMC-1 mast cells[8] and canine BR mastocytoma cells[51]. Therefore, enprofylline can be considered as a selective, though not potent, A_{2B} antagonist.

More potent, but nonselective, A_{2B} receptor antagonists have been also characterized. These compounds include 1,3-dipropyl-8-sulphophenylxanthine (DPSPX), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and xanthine amine congener (XAC)[54,55]. DPSPX has been shown to be 20 times more potent at HEL A_{2B} receptors (K_B = 141 nM) than at platelet A_{2A} receptors[54]. However, the affinities of A_3 receptors in sheep[61] and A_1 receptors in rats[63] for DPSPX are comparable to that of the A_{2B} receptor in HEL cells[54]. It appears that substituents in position 8 of the xanthine nucleus in DPSPX, DPCPX and XAC can confer higher A_{2B} antagonist potency. On the other hand, the lack of 1-alkyl substituents in enprofylline renders this A_{2B} antagonist ineffective at other receptor subtypes. The combination of these properties could provide more potent and selective A_{2B} antagonists although a systematic study of the relationship between structure and activity will first be necessary.

It should be noted that adenosine has conflicting effects on inflammatory processes. The activation of A_{2A} receptors inhibits oxidative burst, degranulation and adhesion of neutrophils[64,65] and also inhibits platelet aggregation[59]. Adenosine at A_1 receptors can increase neutrophil chemotaxis[64] whereas activation of A_3 receptors inhibits eosinophil chemotaxis[52]. Selective block of the putative pro-inflammatory effects of A_{2B} receptors on mast cells might be advantageous. However, it should be remembered that mast cells are only one of many contributors to inflammation in asthma. Therefore, it is unlikely, that a medication targeting a single mechanism will be completely and universally effective in asthma. Nonetheless, selective antagonism of the A_{2B} receptor subtype could help to provide a more potent and safer alternative to theophylline. Greater selectivity of the site of action could be accomplished using an inhaled preparation. More studies are clearly needed to define the advantage of this approach in the treatment of asthma.

Concluding remarks

Abundant evidence has accumulated that responses produced by adenosine are not a mere reflection of nonspecific airways hyperresponsiveness but involve a selective interaction with activated inflammatory cells in diseased airways and might be related to atopy. The airway response to adenosine could be useful in the further exploration of the inflammatory and immunological processes in allergic tissue responses at mucosal surfaces. It is possible that adenosine administered by inhalation could be a promising diagnostic test in asthma[66], but further studies are warranted to define the value of this unique stimulus in following disease activity and in response to therapy. The appreciation of the potential role of A_{2B} receptors in the pathogenesis of asthma raises the possibility that A_{2B} receptors could become the target for future drug development[67]. Progress in the study of A_{2B} receptors will be greatly advanced by the introduction of specific pharmacological probes for this receptor type. Even though adenosine analogues generally have a poor affinity for A_{2B} receptors, this is not the case for antagonists. Hence, the development of potent and selective A_{2B} antagonists might provide a novel approach to the treatment of asthma.

Selected references

1 Belardinelli, L. and Pelleg, A., eds (1995) *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology*, Kluwer Academic Publishers
2 Ongini, E. and Fredholm, B. B. (1996) *Trends Pharmacol. Sci.* 17, 364–372
3 Cushley, M. J., Tattersfield, A. E. and Holgate, S. T. (1983) *Br. J. Clin. Pharmacol.* 83, 161–165
4 Mann, J. S., Holgate, S. T., Renwick, A. G. and Cushley, M. J. (1986) *J. Appl. Physiol.* 86, 1667–1676
5 Crimi, N. *et al.* (1988) *Allergy* 88, 179–183
6 Mann, J. S. and Holgate, S. T. (1985) *Br. J. Clin. Pharmacol.* 19, 685–692
7 Clarke, H., Cushley, M. J., Persson, C. G. and Holgate, S. T. (1989) *Pulm. Pharmacol.* 2, 147–154
8 Feoktistov, I. and Biaggioni, I. (1995) *J. Clin. Invest.* 96, 1979–1986
9 Holgate, S. T., Church, M. K. and Polosa, R. (1991) *Ann. New York Acad. Sci.* 629, 227–236
10 Peachell, P. T., Columbo, M., Kagey-Sobotka, A., Lichtenstein, L. M. and Marone, G. (1988) *Am. Rev. Respir. Dis.* 138, 1143–1151
11 Phillips, G. D., Rafferty, P., Beasley, R. and Holgate, S. T. (1987) *Thorax* 87, 939–945
12 Rafferty, P., Beasley, C. R. and Holgate, S. T. (1987) *Am. Rev. Respir. Dis.* 136, 369–373
13 Crimi, N. *et al.* (1989) *J. Allergy Clin. Immunol.* 83, 921–925
14 Crimi, N. *et al.* (1995) *Eur. Respir. J.* 95, 905–912
15 Phillips, G. D., Ng, W. H., Church, M. K. and Holgate, S. T. (1990) *Am. Rev. Respir. Dis.* 90, 9–13
16 Polosa, R. *et al.* (1995) *Am. J. Respir. Crit. Care Med.* 151, 624–629
17 Crimi, N., Polosa, R. and Mistretta, A. (1993) *Drug Dev. Res.* 28, 322–327
18 Polosa, R. *et al.* (1994) *J. Allergy Clin. Immunol.* 93, 329
19 Gustafsson, L. E., Wiklund, N. P. and Cederqvist, B. (1986) *Eur. J. Pharmacol.* 120, 179–185
20 Pauwels, R. and Van Der Straeten, M. (1987) *Am. Rev. Respir. Dis.* 136, 374–378
21 Polosa, R., Phillips, G. D., Rajakulasingam, K. and Holgate, S. T. (1991) *J. Allergy Clin. Immunol.* 91, 939–947
22 Crimi, N., Palermo, F., Oliveri, R., Polosa, R., Settinieri, I. and Mistretta, A. (1992) *Eur. Respir. J.* 5, 560–565
23 Manzini, S. and Ballati, L. (1990) *Br. J. Pharmacol.* 100, 251–256
24 Polosa, R., Rajakulasingam, K., Church, M. K. and Holgate, S. T. (1992) *Eur. Respir. J.* 92, 700–706
25 Driver, A. G., Kukoly, C. A., Ali, S. and Mustafa, S. J. (1993) *Am. Rev. Respir. Dis.* 148, 91–97
26 Marquardt, D. L., Gruber, H. E. and Wasserman, S. I. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 6192–6196
27 Madara, J. L. *et al.* (1993) *J. Clin. Invest.* 91, 2320–2325
28 Watt, A. H., Penny, W. J., Singh, H., Routledge, P. A. and Henderson, A. H. (1987) *Br. J. Clin. Pharmacol.* 24, 665–668
29 Rose, F. R., Hirschhorn, R., Weissman, G. and Cronstein, B. N. (1988) *J. Exp. Med.* 167, 1186–1194
30 Church, M. K., Holgate, S. T. and Hughes, P. J. (1983) *Br. J. Pharmacol.* 83, 719–726
31 Björck, T., Gustafsson, L. E. and Dahlén, S. E. (1992) *Am. Rev. Respir. Dis.* 145, 1087–1091

32 Phillips, G. D. and Holgate, S. T. (1988) *Clin. Sci.* 88, 429–436
33 Mann, J. S., Cushley, M. J. and Holgate, S. T. (1985) *Am. Rev. Respir. Dis.* 85, 1–6
34 Oosterhoff, Y., de Jong, J. W., Jansen, M. A. M., Koeter, G. H. and Postma, D. S. (1993) *Am. Rev. Respir. Dis.* 147, 553–558
35 Avital, A., Springer, C., Bar-Yishay, E. and Godfrey, S. (1995) *Thorax* 50, 511–516
36 O'Connor, B. J., Ridge, S. M., Barnes, P. J. and Fuller, R. W. (1992) *Am. Rev. Respir. Dis.* 146, 560–564
37 Polosa, R., Rajakulasingam, K., Prosperini, G. and Holgate, S. T. (1993) *Thorax* 48, 324–329
38 van Velzen, E., van den Bos, J-W., Benckhuijsen, J. A. W., van Essel, T., de Bruijn, R. and Aalbers, R. (1996) *Thorax* 51, 582–584
39 Marquardt, D. L., Parker, C. W. and Sullivan, T. J. (1978) *J. Immunol.* 120, 871–878
40 Church, M. K., Hughes, P. J. and Vardey, C. J. (1986) *Br. J. Pharmacol.* 87, 233–242
41 Zhou, Q-Y., Li, C., Olah, M. E., Johnson, R. A., Stiles, G. L. and Civelli, O. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 7432–7436
42 Ramkumar, V., Stiles, G. L., Beaven, M. A. and Ali, H. (1993) *J. Biol. Chem.* 268, 16887–16890
43 Marquardt, D. L., Walker, L. L. and Heinemann, S. (1994) *J. Immunol.* 152, 4508–4515
44 Meade, C. J., Mierau, J., Leon, I. and Ensinger, H. A. (1996) *J. Pharmacol. Exp. Ther.* 279, 1148–1156
45 Hannon, J. P., Pfannkuche, H. J. and Fozard, J. R. (1995) *Br. J. Pharmacol.* 115, 945–952
46 Nyce, J. W. and Metzger, W. J. (1997) *Nature* 97, 721–725
47 Ali, S., Metzger, W. J. and Mustafa, S. J. (1996) *J. Pharmacol. Exp. Ther.* 96, 639–644
48 Marquardt, D. L. and Walker, L. L. (1990) *Biochem. Pharmacol.* 39, 1929–1934
49 Marquardt, D. L. and Walker, L. L. (1994) *Agents Actions* 43, 7–12
50 Feoktistov, I., Sheller, J. R. and Biaggioni, I. (1996) *FASEB J.* 10, A1232 (Abstr.)
51 Auchampach, J. A., Jin, X., Wan, T. C., Caughey, G. H. and Linden, J. (1997) *Mol. Pharmacol.* 52, 846–860
52 Walker, B. A. M. *et al.* (1997) *Am. J. Respir. Cell. Mol. Biol.* 16, 531–537
53 Peachell, P. T., MacGlashan, D. W. J., Lichtenstein, L. M. and Schleimer, R. P. (1988) *J. Immunol.* 140, 571–579
54 Feoktistov, I. and Biaggioni, I. (1993) *Mol. Pharmacol.* 43, 909–914
55 Brackett, L. E. and Daly, J. W. (1994) *Biochem. Pharmacol.* 47, 801–814
56 Fredholm, B. B. and Persson, C. G. (1982) *Eur. J. Pharmacol.* 81, 673–676
57 Lupica, C. R., Cass, W. A., Zahniser, N. R. and Dunwiddie, T. V. (1990) *J. Pharmacol. Exp. Ther.* 252, 1134–1141
58 Ukena, D., Schirren, C. G. and Schwabe, U. (1985) *Eur. J. Pharmacol.* 117, 25–33
59 Ledent, C. *et al.* (1997) *Nature* 388, 674–678
60 Persson, C. G., Andersson, K. and Kjellin, G. (1986) *Life Sci.* 38, 1057–1072
61 Linden, J. *et al.* (1993) *Mol. Pharmacol.* 44, 524–532
62 Robeva, A. S. *et al.* (1996) *Drug Dev. Res.* 39, 243–252
63 Ukena, D., Jacobson, K. A., Kirk, K. L. and Daly, J. W. (1986) *FEBS Lett.* 199, 269–274
64 Cronstein, B. N., Levin, R. I., Philips, M., Hirschhorn, R., Abramson, S. B. and Weissmann, G. (1992) *J. Immunol.* 92, 2201–2206
65 Fredholm, B. B. (1997) *Gen. Pharmacol.* 28, 345–350
66 Polosa, R. and Holgate, S. T. (1997) *Thorax* 52, 919–923
67 Feoktistov, I. and Biaggioni, I. (1997) *Pharmacol. Rev.* 49, 381–402

Chemical name
CGS21680: 4-((*N*-ethyl-5'-carbamoyladenos-2-yl)-aminoethyl)-phenylpropionic acid

Ackowledgements
The authors' work is supported by NIH grants R29HL55596, 1PO1HL56693 and RR00095, and a British Medical Research Council Programme Grant No. PG-860-4034.

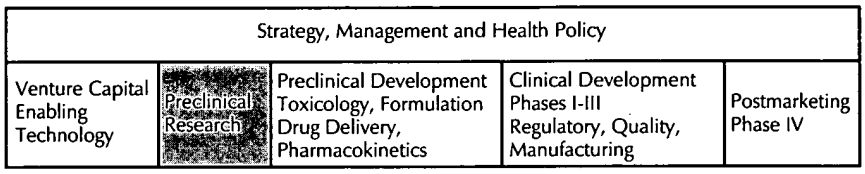
DRUG DEVELOPMENT RESEARCH 45:198–206 (1998)

Research Overview

Adenosine A_{2B} Receptors as Therapeutic Targets

Igor Feoktistov, Jack N. Wells, and Italo Biaggioni*

Divisions of Clinical Pharmacology and Cardiology, Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, Tennessee



ABSTRACT Recent evidence indicates that A_{2B} receptors mediate cellular functions with potential clinical relevance. Both A_{2A} and A_{2B} receptors mediate vasodilation, and the receptor type involved depends on the vascular bed and species studied. In some experimental models, A_{2B}-induced vasodilation is mediated by the endothelium, but it is unclear whether this vasodilation is due to nitric oxide or whether A_{2B} receptors also mediate direct vascular smooth muscle relaxation. A_{2B} receptors expressed in smooth muscle cells inhibit their growth, raising the possibility that these receptors play a role in the vascular remodeling process observed in hypertension and atherosclerosis. A_{2B} receptors are also expressed in neurons, and there are several examples of these receptors mediating neuroexcitatory actions, including potentiation of neurotransmitter release. The highest expression of A_{2B} receptors is found throughout the intestinal tract. During diarrheal processes, neutrophils recruited into intestinal crypts release a soluble "neutrophil-derived secretagogue," which then increases intestinal secretion. It is now known that this neutrophil product is AMP, which is then converted to adenosine to activate A_{2B} receptors expressed in intestinal epithelium. It was also recently found that activation of human mast cells by adenosine is mediated by A_{2B} receptors. Mast cell activation is involved in adenosine-induced bronchoconstriction in asthmatics, suggesting that A_{2B} receptors are involved in this process. Our understanding of the functional role of A_{2B} receptors is hindered by the lack of selective agonist and antagonist of this receptor type. Recent studies suggest the feasibility of developing A_{2B} antagonists. Such agents may prove useful in the treatment of diarrheal diseases and in asthma. Drug Dev. Res. 45:198–206, 1998.

Key words: receptors, purinergic; mast cells; asthma; intracellular pathways; G-coupled receptors

INTRODUCTION

Adenosine is an endogenous nucleoside that modulates many physiologic processes. Its actions are mediated by interaction with specific cell membrane receptors. Four subtypes of adenosine receptors have been cloned: A_1, A_{2A}, A_{2B}, and A_3. Significant advancement has been made in the understanding of the molecular pharmacology and physiologic relevance of adenosine receptors, but our knowledge of A_{2B} receptors lags behind that of other receptor subtypes. The lack of selective pharmacologic probes has hindered research in this area. Perhaps because of their lower affinity for adenosine compared with the other receptors, it is often assumed that A_{2B} receptors are a low-affinity version of the A_{2A} receptor, and of lesser physiologic relevance. It has been only recently that potentially important functions have been discovered for the A_{2B} receptor, prompting a renewed interest in this receptor type. A_{2B} receptors have been implicated in mast cell activation and asthma, vasodilation, regulation of cell growth, intestinal function, and modulation of neurosecretion. We will try to review the recent advances made in the study of A_{2B} receptors, focusing on potential areas in which A_{2B} receptors could be targeted for the development of novel therapeutic agents.

Grant sponsor: National Institutes of Health; Grant number: RR00095; Grant number: HL56693; Grant number: R29HL55596.

*Correspondence to: Italo Biaggioni, Division of Clinical Pharmacology, Vanderbilt University, Nashville, TN 37232-2195. E-mail: itlo.biaggioni@mcmail.vanderbilt.edu

CLASSIFICATION AND MOLECULAR CHARACTERIZATION OF ADENOSINE RECEPTORS

Purinergic receptors that mediate the effects of adenosine are classified as P_1 receptors, whereas the receptors activated by nucleotides such as ATP are classified as P_2 receptors. Adenosine receptors were initially subdivided into A_1 and A_2 subtypes, based on their ability to inhibit or stimulate adenylyl cyclase, respectively. The further division of A_2 receptors into two subtypes was based on the finding of high-affinity A_2 receptors in rat striatum and low-affinity A_2 receptors throughout the brain, both of which activated adenylyl cyclase [Daly et al., 1983], and the existence of high-affinity A_2 receptors in cultured neuroblastoma cells and low-affinity A_2 receptors in glioma cells [Elfman et al., 1984]. These high- and low-affinity receptor subtypes were later designated A_{2A} and A_{2B}, respectively [Bruns et al., 1986]. The classification of P_1 receptors has been validated by the recent success in molecular cloning and expression of all three anticipated A_1, A_{2A}, and A_{2B} adenosine receptors and the previously unrecognized A_3 receptor.

Adenosine A_{2B} receptors have been cloned from rat hypothalamus [Rivkees and Reppert, 1992], human hippocampus [Pierce et al., 1992], and mouse mast cells [Marquardt et al., 1994]. The proposed membrane structure of A_{2B} receptors is typical of G-protein–coupled receptors, with seven transmembrane domains connected by three extracellular and three intracellular loops [Rivkees and Reppert, 1992; Pierce et al., 1992; Marquardt et al., 1994]. The predicted molecular mass of A_{2B} receptors is similar to that of A_1 and A_3 receptors (36–37 kDa), whereas A_{2A} receptors have a larger predicted size (45 kDa). The human A_{2B} receptor gene was mapped to chromosome 17p11.2-p12 [Townsend-Nicholson et al., 1995; Jacobson et al., 1995]. A single intron interrupts the coding sequence of the human A_{2B} receptor gene in a region corresponding to the second intracellular loop [Jacobson et al., 1995]. Recent findings indicate that A_{2B} receptors can act as anchoring proteins for adenosine deaminase, as previously shown for A_1 receptors [Ciruela et al., 1996]. It is not known which portions of the receptor are involved in this process and whether they are shared by both receptors. It is also not clear the functional relevance of this phenomenon. It can be speculated that the proximity of adenosine deaminase to the receptor may serve as a mechanism to terminate adenosine's actions.

PHARMACOLOGY OF A_{2B} RECEPTORS AND INTRACELLULAR PATHWAYS

Highly selective and potent agonists have been designed for A_1, A_{2A}, and A_3 receptors. Each adenosine receptor has a typical order of potency for agonists (Table 1). However, no potent and selective agonist for A_{2B} receptors has been found so far. The adenosine analog 5′-*N*-ethylcarboxamidoadenosine (NECA) remains the most potent A_{2B} agonist [Bruns, 1981; Brackett and Daly, 1994; Feoktistov and Biaggioni, 1993, 1998], with an EC_{50} for stimulation of adenylyl cyclase of approximately 2 μM. NECA, however, activates other adenosine receptors with even greater affinity, with an EC_{50} in the low nanomolar (A_1 and A_{2A}) or high nanomolar (A_3) range (Table 1). The characterization of A_{2B} receptors, therefore, often relies on a method of exclusion, i.e., by the lack of effectiveness of compounds that are potent and selective agonists of other receptor types. A_{2B} receptors have a very low affinity for the A_{2A}-selective agonist 4-((*N*-ethyl-5′-carbamoyladenos-2-yl)-aminoethyl)-phenylpropionic acid (CGS 21680) [Jarvis et al., 1989; Nakane and Chiba, 1990; Webb et al., 1992; Hide et al., 1992; Feoktistov and Biaggioni, 1993; Alexander et al., 1996], the A_1-selective agonist R-PIA [Feoktistov and Biaggioni, 1993; Brackett and Daly, 1994], as well as for the A_3-selective agonist IB-MECA [Feoktistov and Biaggioni, 1998]. The agonist profile NECA

TABLE 1. Pharmacological Characteristics of Adenosine Receptor Subtypes[a]

Adenosine receptor	Order of potency for agonists[b] (μM)	Selective agonists[c]	Selective antagonists[c]
A_1	R-PIA (0.001) > NECA (0.006) > IB-MECA (0.054) > CGS 21680 (2.6)	R-PIA CPA	DPCPX N-0861
A_{2A}	NECA (0.01) = CGS 21680 (0.015) > IB-MECA (0.056) > R-PIA (0.124)	CGS 21680 APEC	SCH 58261 ZM 241385
A_{2B}	NECA (2) > R-PIA (160) = IB-MECA (201) > CGS 21680 (1600)	None	Enprofylline
A_3	IB-MECA (0.001) > NECA (0.113) = R-PIA (0.158) > CGS 21680 (0.584)	IB-MECA Cl-IB-MECA	MRS 1067 MRS 1097 L-249313 L-268605

[a]Table reproduced from Feoktistov and Biaggioni [1997], with permission.

[b]Data shown for rat A_1, A_{2A}, and A_3 receptors are K_i values based on radioligand binding [van Galen et al., 1994; Gallo-Rodriguez et al., 1994]. Data shown from A_{2B} receptors are EC_{50} values for cAMP accumulation in human erythroleukemia cells [Feoktistov and Biaggioni, 1993; Feoktistov and Biaggioni, 1998].

[c]Data are derived from Feoktistov and Biaggioni [1995], Palmer and Stiles [1995], Jacobson and Suzuki [1996], and Jacobson [1996].

> R-PIA = IB-MECA > CGS 21680, determined in human erythroleukemia cells, is typical for A_{2B}-mediated cAMP accumulation. Given this rank order of potency, responses elicited by NECA at concentrations in the low micromolar range (1–10 μM), but not by R-PIA, IB-MECA, or CGS 21680, are characteristic of A_{2B} receptors.

Pharmacologic characterization of receptors based on apparent agonist potencies, however, is far from ideal because it depends not only on agonist binding to the receptor but also on multiple processes involved in signal transduction. Selective antagonists are preferable for receptor subtype identification. Highly selective and potent A_{2B} antagonists are not yet available, but, whereas A_{2B} receptors have a lower affinity for agonists compared with other receptor subtypes, this is not true for antagonists. At least some xanthines are as potent antagonists at A_{2B} receptors as at other adenosine receptors [Feoktistov and Biaggioni, 1993; Brackett and Daly, 1994].

The antiasthmatic drug enprofylline (3-*n*-propylxanthine) is the most selective A_{2B} antagonist known to date. In early studies, enprofylline was found to be approximately 20 times more potent in blocking hippocampal A_2 receptors compared with rat fat cell A_1 receptors [Fredholm and Persson, 1982]. It was initially proposed, therefore, that enprofylline can selectively block a subtype of A_2 receptors in the hippocampus [Fredholm and Persson, 1982]. However, enprofylline was then found to be a poor antagonist of A_2 receptors in thymocytes [Fredholm and Sandberg, 1983] and in platelets [Ukena et al., 1985], leading the authors to conclude that enprofylline was not an adenosine receptor antagonist. However, it is now known that accumulation of cAMP in hippocampal slices, which was shown to be blocked by enprofylline, is mediated by A_{2B} receptors [Lupica et al., 1990], and that platelets, found to be insensitive to enprofylline, express mainly A_{2A} receptors [Feoktistov and Biaggioni, 1993; Dionisotti et al, 1996; Ledent et al., 1997]. Therefore, previous contradictory results can now be explained by a selective antagonism of A_{2B} receptors by enprofylline. Indeed, it has since been demonstrated that enprofylline is equipotent to theophylline as an A_{2B} receptor antagonist in human erythroleukemia cells (HEL), with a K_B of 7 μM [Feoktistov and Biaggioni, 1995]. This finding has been confirmed in subsequent studies (Table 2). More potent A_{2B} receptor antagonists have been also characterized, including DPSPX, DPCPX, and XAC [Feoktistov and Biaggioni, 1993; Brackett and Daly, 1994]. These antagonists, however, are not specific for A_{2B} receptors. For example, the affinity of DPSPX for A_{2B} receptors is similar to those of sheep A_3 [Linden et al., 1993] and rat A_1 [Ukena et al., 1986] receptors. Among nonxanthine compounds, alloxazine was reported to be ninefold more potent as an A_{2B} receptors antagonist in VA13 and NIH 3T3 cells compared with A_{2A} receptors in PC12 cells [Brackett and Daly, 1994].

Both A_{2A} and A_{2B} receptors are coupled to G_S proteins and activate adenylyl cyclase in virtually every cell where they are expressed. Although activation of adenylyl cyclase is arguably an important signaling mechanism for A_{2A} receptors, this is not necessarily the case for A_{2B} receptors, because other intracellular signaling pathways have been found to be functionally coupled to A_{2B} receptors in addition to adenylyl cyclase. A_{2B} receptors have been shown to be coupled to phospholipase C in *Xenopus* oocytes expression system [Yakel et al., 1993], in mouse bone marrow-derived mast cells [Marquardt et al., 1994], and, through G_q proteins, in human mast HMC-1 cells [Feoktistov and Biaggioni, 1995] and canine BR mastocytoma cells [Auchampach et al., 1997]. A_{2B} receptor-mediated stimulation of β-phospholipase C results in mobilization of intracellular calcium in HMC-1 cells and eventually promotes synthesis of IL-8 [Feoktistov and Biaggioni, 1995], possibly through stimulation of MAP kinases [Feoktistov et al., 1998b]. Stimulation of A_{2B} receptors also increases intracellular calcium in HEL cells through a cholera toxin–sensitive but cAMP-independent mechanism. It has been suggested that αG_S, coupled to A_{2B} receptors, can directly stimulate a putative calcium channel [Feoktistov et al., 1994].

Activation of A_{2B} receptors can also increase intracellular calcium by potentiating P-type calcium currents in pyramidal neurons from the CA3 region of guinea pig hippocampus. This mechanism was thought to be mediated by adenylyl cyclase because this potentiation could be inhibited by blocking the cAMP-dependent protein kinase [Mogul et al., 1993].

There are now several well-documented examples of cells coexpressing both A_{2A} and A_{2B} receptors. The functional significance of this phenomenon has been unclear, because A_{2A} receptors have a much higher affinity for the endogenous agonist adenosine than A_{2B} receptors. The existence of differential intracellular pathways for A_{2A} and A_{2B} receptors suggests that activation of these receptors may have independent functional roles even when ex-

TABLE 2. Evidence for Enrpofylline as an A_{2B} Antagonist

Tissue	Method	Potency	Reference
Rat hippocampal slices	cAMP accumulation (NECA)	K_B = 6 μM	Fredholm and Persson, 1982
Human Erythroleukemia cells	cAMP accumulation (NECA)	K_B = 7 μM	Feoktistov and Biaggioni, 1995
CHO cells expressed with human A_{2B} receptors	[^{3}H]DPX binding	K_i = 7 μM	Robeva et al., 1996

pressed in the same cells. An unresolved question is whether these receptors interact with each other. The human mast cell line HMC-1 can be used as an example; these cells express both A_{2A} and A_{2B} receptors, but only A_{2B} receptors activate these cells to increase interleukin-8 production by means of mitogen-activated protein (MAP) kinases. Selective activation of A_{2A} receptors, while inducing cAMP accumulation, had no apparent effect on HMC-1 cell function. Preliminary evidence, however, suggest that A_{2A} receptors are required, and are even essential, for A_{2B}-induced MAP kinase activation [Feoktistov et al., 1998a,b]. These findings raise the possibility that A_{2A} and A_{2B} receptors interact in a cooperative manner. It is now known, however, if this phenomena is limited to HMC-1 cells or if it shared by other cells coexpressing A_{2A} and A_{2B} receptors.

PHYSIOLOGIC RELEVANCE OF A_{2B} RECEPTORS

Modulation of Vascular Tone

Adenosine-induced vasodilation has been traditionally attributed to activation of A_{2A} receptors. However, there are vascular beds in which the nonselective agonist NECA produces profound vasodilation, but the selective A_{2A} agonist CGS 21680 has little effect, suggesting the involvement of A_{2B} receptors [Webb et al., 1992]. This phenomenon is observed in guinea pig aorta and dog saphenous vein [Hargreaves et al., 1991] and in dog coronary arteries [Balwierczak et al., 1991]. Both A_{2A} and A_{2B} receptors may mediate vasodilation in the same species. In guinea pig, for instance, A_{2A} receptors mediate relaxation of coronary vessels, whereas A_{2B} receptors produce vasodilation of the aorta [Martin, 1992; Martin et al., 1993].

The vasodilatory effects of adenosine can be explained by direct vascular smooth muscle relaxation. However, recent studies have suggested that the endothelium contributes to, or is even essential for, the vasodilatory effects of intravascular adenosine. In this regard, A_{2B} receptors are present in endothelial cells. Both A_{2B} and A_{2A} receptors regulate cAMP production in human aortic [Iwamoto et al., 1994] and human umbilical vein endothelial cells [Feoktistov et al., 1998c], and A_{2B} receptor mRNA has been detected in human aortic endothelial cells [Iwamoto et al., 1994]. Few studies have directly examined the possible interaction between A_{2B} receptors and endothelium-derived vasodilation, and results vary depending on the vascular bed studied. A_{2B} receptors mediate vasodilation independent of nitric oxide generation in the rat mesenteric arterial bed [Rubino et al., 1995] and in the isolated blood-perfused rat lung preparation [Haynes et al., 1995]. On the contrary, isolated rat renal artery rings contain A_{2B} receptors that are located exclusively on the endothelium and cause nitric oxide release and vasodilation [Martin and Potts, 1994]. Similarly, A_{2B} receptors also appear to vasodilate the rabbit corpus cavernosum, and this effect is reduced by removal of the endothelium [Chiang et al., 1994].

Whereas most studies of the cardiovascular effects of adenosine have focused on its acute actions on vascular tone, recent evidence suggests that adenosine may also play a long-term modulatory role on smooth muscle growth. A_{2B} receptors inhibit rat aortic smooth muscle cell growth induced by fetal calf serum [Dubey et al., 1996]. Activation of adenylyl cyclase is postulated as the signaling pathway involved because this effect is mimicked by 8-bromo-cAMP. It was also shown that stimulation of vascular smooth muscle cell growth by fetal calf serum also triggers release of endogenous adenosine, which then acts in an autocrine fashion to inhibit growth [Dubey et al., 1996a,b]. If a role for endogenous adenosine is confirmed, this finding would establish a novel cardioprotective effect of adenosine, with relevance to vascular remodeling processes observed in hypertension and atherosclerosis. On the other hand, preliminary reports implicate A_{2B} receptors in mediating human retinal endothelial cell growth by inducing the vascular endothelial growth factor (VEGF) [Grant et al., 1998].

Modulation of Neurosecretion and Neurotransmission

A_{2B} receptors are widespread in the brain, but little is known about their function. There are, however, several examples of A_{2B} receptors mediating neuroexcitatory actions. Adenosine agonists increase the release of the excitatory amino acid aspartate in rat cerebral cortex cup superfusates in vivo, while depressing the release of the inhibitory amino acid γ-aminobutyric acid [Phillis et al., 1993b], with an agonist profile suggestive of an A_{2B} receptor. A_{2B} receptors also enhance basal release of acetylcholine in this same model [Phillis et al., 1993a]. Adenosine agonists potentiate a P-type Ca^{2+} current in pyramidal neurons from the CA3 region of guinea pig hippocampus with a pharmacologic profile consistent with A_{2B} receptors [Mogul et al., 1993]. Likewise, A_{2B} receptors induce long-term potentiation in the CA1 region of rat hippocampus [Kessey et al., 1997]. The importance of these phenomena is unclear, because they are only observed after blockade of A_1 receptors, which is also present in these hippocampal cells.

Adenosine also modulates release of catecholamines from chromaffin cells, probably through A_{2B} receptors [Casado et al., 1992]. At high concentrations (20 μm), the nonselective agonist NECA inhibits catecholamine release induced by nicotinic stimulation, presumably by activation of A_{2B} receptors [Mateo et al., 1995]. This effect has an unusually slow time course and is seen only after 20–30 min of preincubation with NECA; its physiological relevance is not clear.

Regulation of Intestinal Tone and Secretion

The high levels of A_{2B} receptor expression found in different parts of the intestinal tract motivated great interest in defining their function. Adenosine elicits relaxation of dispersed guinea pig longitudinal muscle cells from small intestine by means of A_{2B} receptors coupled to adenylyl cyclase [Murthy et al., 1995]. The A_{2B}-mediated relaxation was evident only after A_1 receptor blockade, raising doubts as to their importance. However, blockade of A_2 receptors potentiated A_1-mediated contraction, indicating that A_{2B} receptors do provide a restraining function against intestinal contraction. In rat duodenum A_{2B} receptors cause relaxation of longitudinal muscle, but contraction of muscularis mucosae [Nicholls et al., 1996]. This is an unexpected result and the first example of an excitatory response by A_{2B} receptors in a smooth muscle preparation. A_{2B} receptors have also been shown to relax guinea pig *Taenia caeci* [Prentice and Hourani, 1997]. The functional relevance of the intestinal relaxant actions of A_{2B} receptors has not been defined.

The effect of A_{2B} receptors on epithelial secretion has received particular attention because of its potential relevance to diarrheal processes. As part of the pathophysiology of these disorders, neutrophils are recruited into intestinal crypts where they release a soluble "neutrophil-derived secretagogue" that then activates intestinal epithelium to stimulate chloride secretion, an important mechanism of diarrheal diseases. This neutrophil-derived secretagogue has recently been identified as AMP, which is then converted to adenosine at the epithelial cell surface to stimulate chloride secretion [Madara et al., 1993] by means of activation of A_{2B} receptors [Strohmeier et al., 1995].

Adenosine and Asthma

Adenosine has been implicated in the pathophysiology of asthma (for review see [Church and Holgate, 1986; Feoktistov and Biaggioni, 1996; Feoktistov et al., 1998d]), and several lines of evidence support this hypothesis. Inhaled adenosine, or its precursor AMP, provokes bronchoconstriction in asthmatic patients but not in normal subjects [Cushley et al., 1984]. Dipyridamole, a drug that blocks adenosine uptake and increases its extracellular concentrations, can also produce severe bronchospasm in asthmatic patients [Eagle and Boucher, 1989]. Moreover, theophylline provides a better protection against adenosine-induced bronchoconstriction, than against histamine-induced bronchoconstriction [Mann and Holgate, 1985].

The mechanism by which adenosine produces bronchoconstriction has been the focus of recent interest. Adenosine produces a direct constrictor action on isolated guinea pig trachea by means of A_1 receptors [Ghai et al., 1987]. A_1 receptors also mediate bronchoconstriction in an allergic rabbit model in vivo [Ali et al., 1994a,b], and treatment with antisense oligodeoxynucleotide targeting the adenosine A_1 receptor desensitized the allergic rabbits to subsequent challenge with either adenosine or allergen [Nyce and Metzger, 1997]. A_1 receptors also constrict human bronchi isolated from asthmatics in vitro, but not bronchi isolated from normal subjects [Björck et al., 1992].

The bronchoconstriction produced by inhaled adenosine in humans does not seem to be a direct bronchoconstrictive effect, but rather seems to be mediated through mast cell activation, because it can be blocked by specific antihistamines [Phillips et al., 1987; Rafferty et al., 1987] and prevented by the mast cell inhibitors chromoglycate and nedocromil sodium [Phillips et al., 1989]. Furthermore, inhaled adenosine induces a significant rise in plasma levels of histamine [Phillips et al., 1990] and increases bronchoalveolar levels of histamine, PGD_2, and tryptase in asthmatics but not in normal subjects [Polosa et al., 1995].

Marquardt et al. [1978] were the first to report that adenosine potentiated activation of isolated rat mast cells. It was initially proposed that this effect was mediated by A_3 receptors, because A_3 receptors also activate rat basophilic leukemia RBL-2H3 cells [Ramkumar et al., 1993], a model for the study of rat mast cells. On the other hand, A_{2B} receptors have been shown to potentiate activation of mouse bone marrow–derived mast cells [Marquardt and Walker, 1990]. A_{2B} receptors have been shown to activate the human mast cell line HMC-1 [Feoktistov and Biaggioni, 1995] and canine BR mastocytoma cells [Auchampach et al., 1997]. Adenosine analogs potentiate immunologic activation of human lung mast cells [Peachell et al., 1988], with an order of potency consistent with A_{2B} receptors.

A_{2B} RECEPTORS AS THERAPEUTIC TARGETS

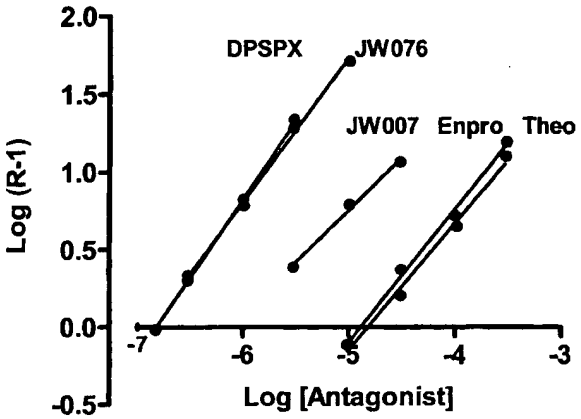
The current therapeutic uses of adenosine are based on its cardiovascular actions. Adenosine has become the drug of choice for the termination of supraventricular arrhythmias, based on its ability to delay atrioventricular node conduction [Bellardinelli et al., 1989]. Intravenous adenosine is used also as a stress test in the diagnosis of myocardial ischemia [Verani et al., 1990] and as a hypotensive agent during anesthesia [Sollevi et al., 1984]. However, adenosine has been implicated in many other physiologic and pathologic processes in addition to its cardiovascular actions. The biggest problem in translating this knowledge into therapeutic tools is perhaps the ubiquity of adenosine receptors, which often mediate contrasting effects. The challenge, therefore, is to develop drugs targeted to a specific receptor. The ongoing development of selective agonists or antagonist represents a

substantial advancement toward this goal. Nonetheless, even if specific agents can be developed for a given receptor subtype, the problem remains of selective targeting the site of action. For example, A_1-selective agonists could be developed for their anti-lipolytic potential. If given systemically, however, it is possible that other A_1-mediated effects, such as atrioventricular conduction delay or bradycardia, may be an undesirable and perhaps limiting effect. In the development of useful therapeutic agents, therefore, care should be taken not only in the targeting of the receptor subtype, but also the site of action. This problem, of course, is not limited to adenosinergic systems and is common to others characterized by the widespread nature of their receptors.

Given that the functional role of A_{2B} receptors is only now being addressed, a discussion of potential therapeutic opportunities arising from modulation of such receptors is necessarily speculative. There are, however, some promising areas that deserve further attention. The potential role of A_{2B} receptors in asthma can be used as an example. If confirmed, this mechanism would provide a novel approach for the treatment of this condition. Asthma continues to be a substantial medical problem that affects approximately 5–7% of the population. Despite advances in its treatment, the prevalence of asthma, emergency department visits, hospitalizations, and mortality related to the disease, all appear to be on the rise. Theophylline continues to be an effective treatment in the prevention of asthma attacks, but considerable plasma levels of approximately 50 μM are needed for it to be effective. Moreover, it has many side effects, which can be attributed to its nonspecificity. For example, its central actions contribute to theophylline's side effect profile and are of doubtful benefit for the treatment of asthma.

If indeed blockade of A_{2B} receptors contributes to the antiasthmatic effects of theophylline, it would be possible to develop selective antagonists for this receptor subtype. Lipophobic compounds would have the advantage of not crossing the blood brain barrier. Specific targeting to the site of action can also be accomplished if compounds are developed that can be administered by inhalation. This proposition is not unrealistic. For example, the xanthine antagonist DPSPX is approximately 100-fold more potent than theophylline as an A_{2B} receptor antagonist. Because DPSPX exists as a negatively charged molecule at physiologic pH, this water-soluble xanthine does not penetrate cell membranes or cross the blood-brain barrier [Tofovic et al., 1991]. It appears that the ionic *p*-sulfophenyl substituent in DPSPX (1,3-dipropyl-8-*p*-sulfophenylxanthine) may confer high A_{2B} potency. The lack of *l*-alkyl substituents in the enprofylline molecule (3-*n*-propylxanthine) renders it an ineffective antagonist of other adenosine receptor subtypes. The systematic study of the structure activity relationship for blockade of A_{2B} receptors, considering the above-mentioned properties, could result in more potent and specific agents. Similarly, A_{2B} receptor antagonists can be developed for the treatment of diarrheal processes, if adenosine is confirmed to play a role in this process. Targeting to the site of action could be achieved with compounds that are poorly absorbed, as long as they are able to reach the intestinal crypts involved in intestinal inflammatory processes.

Our laboratory has recently undertaken a systematic study of xanthine derivatives as A_{2B} receptor antagonists. We used HEL cells as a cellular model, because they seem to express only A_{2B} receptors, and used cAMP accumulation as evidence of A_{2B} receptor activation. We believe this approach is as effective as the alternative of using radioligand binding in cells overexpressing A_{2B} receptors. Both approaches have provided virtually identical estimates of the potency of enprofylline as an A_{2B} antagonist (Table 2). After an initial screening of over 100 compounds, 21 were selected for more detailed evaluation. Of these, two seem particularly promising (Fig. 1). JW-076 was the most potent A_{2B} antagonist studied, with



Antagonist Potency (μM)

	A1	A2A	A2B	A3
DPSPX	0.140	0.800	0.140	
JW-076	0.100	>3	0.135	?
JW-007	14	>100	0.770	?
Enprofylline	156	32	7	65
Theophylline	8.5	25	12	86

Fig. 1. Antagonistic effects of xanthine derivatives on A_{2B} receptors. Figure shows Schild analysis derived from dose-response curves for accumulation of cAMP produced by NECA in human erythroleukemia cells in the absence and in the presence of increasing concentrations of the antagonists. Schild analysis revealed a linear relationship for all compounds, suggesting competitive antagonism at A_{2B} receptors. The intercept to the x-axis is an estimate of the K_i of the antagonist.

an IC_{50} of 135 nM. It has a low affinity to A_{2A} receptors (IC_{50} > 3 μM) and may be useful in differentiating between A_2 receptor subtypes, but it is an effective A_1 antagonist, with a K_i of 100 nM. On the other hand, JW-007 seems to be a more selective A_{2B} antagonist, with an IC_{50} of 770 nM for A_{2B} receptors. It has a much lower affinity for A_1 receptors (K_i of 14 μM) and is virtually ineffective at A_{2A} receptors (K_i > 100 μM).

Zwart et al. synthesized C5 substituents of the A_{2A} antagonist ZM241385 and found that the benzylamino substitute analog was an effective A_{2B} antagonist, with an IC_{50} for A_{2B}-mediated adenylyl cyclase activation of 500 nM, and an K_i for inhibition of A_{2B} receptor binding of 7.7 nM. This compound, however, is nonselective, because it is equally potent at A_{2A} receptors [Zwart et al., 1998]. Derivatives of the nonselective adenosine receptor antagonist CGS 15943 were also found to be effective A_{2B} antagonists with an IC_{50} of 270 nM, but were nonselective [Kim et al., 1998]. Similarly, systematic substitutions at the 2-, 6-, and 8-positions of the purine ring have been made with the aim of developing A_{2B} antagonists. Of these, 8-bromo-9-ethyladenine showed a K_i of 840 nM [Vittori et al., 1998]. It is too early to define the potential therapeutic relevance of these or related compounds, but these results at least demonstrate the feasibility of developing potent and selective A_{2B} antagonists.

Development of agonists to target A_{2B} receptors, e.g., to inhibit vascular smooth muscle growth, would be a greater challenge. Substantial progress would need to be made to develop a potent enough agonist that would selectively activate the low affinity A_{2B} receptor while having negligible actions at other receptors. The observation that A_{2B} receptors mediate vasodilation of the rabbit corpus cavernosum [Chiang et al., 1994] raises the possibility that agonist to this receptor type can be useful in impotence. Direct injections of adenosine into the corpus cavernosum of impotence patients produces a brief erection [Kilic et al., 1994], particularly if combined with prostaglandin E_1 [Chiang et al., 1994]. The brief duration of effect is clearly related to the short half-life of adenosine in humans [Moser et al., 1989]. If this effect is also mediated by A_{2B} receptors in humans, it will be possible to develop stable and selective agonists that can be given locally.

REFERENCES

Alexander SPH, Cooper J, Shine J, Hill SJ. 1996. Characterization of the human brain putative A_{2B} receptor expressed in Chinese hamster ovary ($CHO.A_{2B4}$) cells. Br J Pharmacol 119:1286–1290.

Ali S, Mustafa SJ, Metzger MJ. 1994a. Adenosine receptor-mediated bronchoconstriction and bronchial hyperresponsiveness in allergic rabbit model. Am J Physiol 94:L271–L277.

Ali S, Mustafa SJ, Metzger WJ. 1994b. Adenosine-induced bronchoconstriction and contraction of airway smooth muscle from allergic rabbits with late-phase airway obstruction: evidence for an inducible adenosine A1 receptor. J Pharmacol Exp Ther 94:1328–1334.

Auchampach JA, Jin X, Wan TC, Caughey GH, Linden J. 1997. Canine mast cell adenosine receptors: cloning and expression of the A_3 receptors and evidence that degranulation is mediated by the A_{2B} receptor. Mol Pharmacol 52:846–860.

Balwierczak JL, Sharif R, Krulan CM, Field FP, Weiss GB, Miller MJ. 1991. Comparative effects of a selective adenosine A2 receptor agonist, CGS 21680, and nitroprusside in vascular smooth muscle. Eur J Pharmacol 196:117–123.

Bellardinelli L, Linden J, Berne RM. 1989. The cardiac effects of adenosine. Prog Cardiovasc Dis 32:73–97.

Björck T, Gustafsson LE, Dahlën SE. 1992. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. Am Rev Respir Dis 145:1087–1091.

Brackett LE, Daly JW. 1994. Functional characterization of the A2b adenosine receptor in NIH 3T3 fibroblasts. Biochem Pharmacol 47:801–814.

Bruns RF. 1981. Adenosine antagonism by purines, pteridines and benzopteridines in human fibroblasts. Biochem Pharmacol 30:325–333.

Bruns RF, Lu GH, Pugsley TA. 1986. Characterization of the A2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol Pharmacol 29:331–346.

Casado V, Casillas T, Mallol J, Canela EI, Lluis C, Franco R. 1992. The adenosine receptors present on the plasma membrane of chromaffin cells are of the A2b subtype. J Neurochem 59:425–431.

Chiang PH, Wu SN, Tsai EM, Wu CC, Shen MR, Huang CH. 1994. Adenosine modulation of neurotransmission in penile erection. Br J Clin Pharmacol 38:357–362.

Church MK, Holgate ST. 1986. Adenosine and asthma. Trends Pharmacol Sci 7:49–50.

Ciruela F, Saura C, Canela EI, Mallol J, Lluis C, Franco R. 1996. Adenosine deaminase affects ligand-induced signalling by interacting with cell surface adenosine receptors. FEBS Lett 380:219–223.

Cushley MJ, Tattersfield AE, Holgate ST. 1984. Adenosine-induced bronchoconstriction in asthma. Am Rev Respir Dis 129:380–384.

Daly JW, Butts-Lamb P, Padgett W. 1983. Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. Cell Mol Neurobiol 3:69–80.

Dionisotti S, Ferrara S, Molta C, Zocchi C, Ongini E. 1996. Labeling of A2A adenosine receptors in human platelets by use of the new nonxanthine antagonist radioligand [^{3}H]SCH 58261. J Pharmacol Exp Ther 278:1209–1214.

Dubey RK, Gillespie DG, Mi Z, Suzuki F, Jackson EK. 1996a. Smooth muscle cell-derived adenosine inhibits cell growth. Hypertension 27:766–773.

Dubey RK, Gillespie DG, Osaka K, Suzuki F, Jackson EK. 1996b. Adenosine inhibits growth of rat aortic smooth muscle cells. Possible role of A2b receptor. Hypertension 27:786–793.

Eagle KA, Boucher CA. 1989. Intravenous dipyridamole infusion causes severe bronchospasm in asthmatic patients. Chest 95:258–289 (editorial).

Elfman L, Lindgren E, Walum E, Fredholm BB. 1984. Adenosine analogues stimulate cyclic AMP-accumulation in cultured neuroblastoma and glioma cells. Acta Pharmacol Toxicol 55:297–302.

Feoktistov I, Biaggioni I. 1993. Characterization of adenosine recep-

tors in human erythroleukemia cells. Further evidence for heterogeneity of adenosine A2 receptors. Mol Pharmacol 43:909–914.

Feoktistov I, Biaggioni I. 1995. Adenosine A2b receptors evoke interleukin-8 secretion in human mast cells: an enprofylline-sensitive mechanism with implications for asthma. J Clin Invest 96:1979–1986.

Feoktistov I, Biaggioni I. 1996. Role of adenosine in asthma. Drug Dev Res 39:333–336.

Feoktistov I, Biaggioni I. 1997. Adenosine A_{2B} receptors. Pharmacol Rev 49:381–402.

Feoktistov I, Biaggioni I. 1998. Pharmacological characterization of adenosine A_{2B} receptors: studies in human mast cells co-expressing A_{2A} and A_{2B} adenosine receptor subtypes. Biochem Pharmacol 55:627–633.

Feoktistov I, Murray JJ, Biaggioni I. 1994. Positive modulation of intracellular Ca2+ by adenosine A2b, prostacyclin and prostaglandin E1 receptors via a cholera toxin-sensitive mechanism in human erythroleukemia cells. Mol Pharmacol 45:1160–1167.

Feoktistov I, Goldstein AE, Biaggioni I. 1998a. A_{2A} receptors are essential for A_{2B} receptor function in human mast cells. Drug Dev Res 43:11.

Feoktistov I, Goldstein AE, Biaggioni I. 1998b. A_{2B} receptors stimulate MAP kinase in human mast cells. Drug Dev Res 43:10.

Feoktistov I, Nastanski F, Hoover R, Biaggioni I. 1998c. Adenosine A_{2A} and A_{2B} receptors are expressed in human endothelial cells. Drug Dev Res 43:44.

Feoktistov I, Polosa R, Holgate ST, Biaggioni I. 1998d. Adenosine A_{2B} receptors: a novel therapeutic target in asthma? Trends Pharmacol Sci 19:148–153.

Fredholm BB, Persson CG. 1982. Xanthine derivatives as adenosine receptor antagonists. Eur J Pharmacol 81:673–676.

Fredholm BB, Sandberg G. 1983. Inhibition by xanthine derivatives of adenosine receptor-stimulated cyclic adenosine 3′-5′-monophosphate accumulation in rat and guinea-pig thymocytes. Br J Pharmacol 80:639–644.

Gallo-Rodriguez C, Ji X-D, Melman N, Siegman BD, Sanders LH, Orlinda J, Fischer B, Pu Q, Olah ME, van Galen PJM, Stiles GL, Jacobson KA. 1994. Structure-activity relationship of N^6-benzyladenosine-5′-uronamides as A_3-selective adenosine agonists. J Med Chem 37:636–646.

Ghai G, Zimmerman MB, Hopkins MF. 1987. Evidence for A1 and A2 adenosine receptors in guinea pig trachea. Life Sci 41:1215–1224.

Grant MB, Tarnuzzer RW, Caballero S, Ozeck MJ, Shryock JC, Belardinelli L. 1998. Adenosine (Ado) mediates vascular endothelial growth factor (VEGF) induction through A_{2B} ADO receptor (AdoR) in human retinal endothelial cells (HREC). Drug Dev Res 43:14.

Hargreaves MB, Stoggall SM, Collis MG. 1991. Evidence that the adenosine receptor mediating relaxation in dog lateral saphenous vein and guinea pig aorta is of the A_{2B} subtype. Br J Pharmacol 102:198P (abstract).

Haynes JJ, Obikao B, Thompson WJ, Downey J. 1995. Adenosine-induced vasodilation: receptor characterization in pulmonary circulation. Am J Physiol 268:H1862–H1868.

Hide I, Padgett WL, Jacobson KA, Daly JW. 1992. A2A adenosine receptors from rat striatum and rat pheochromocytoma PC12 cells: characterization with radioligand binding and by activation of adenylate cyclase. Mol Pharmacol 41:352–359.

Iwamoto T, Umemura S, Toya Y, Uchibori T, Kogi K, Takagi N, Ishii M. 1994. Identification of adenosine A2 receptor-cAMP system in human aortic endothelial cells. Biochem Biophys Res Commun 199:905–910.

Jacobson KA. 1996. Molecular diversity in the search for selective ligands for A3-adenosine and ATP receptors. Drug Dev Res 37:112–112.

Jacobson KA, Suzuki F. 1996. Recent developments in selective agonists and antagonists acting at purine and pyrimidine receptors. Drug Dev Res 39:289–300.

Jacobson MA, Johnson RG, Luneau CJ, Salvatore CA. 1995. Cloning and chromosomal localization of the human A2b adenosine receptor gene (ADORA2B) and its pseudogene. Genomics 27:374–376.

Jarvis MF, Schulz R, Hutchinson AJ, Do UH, Sillis MA, Williams M. 1989. [^{3}H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. J Pharmacol Exp Ther 251:888–893.

Kessey K, Trommer BL, Overstreet LS, Ji T, Mogul DJ. 1997. A role for adenosine A_2 receptors in the induction of long-term potentiation in the CA1 region of rat hippocampus. Brain Res 756:184–190.

Kilic S, Salih M, Anafarta K, Baltaci S, Kosar A. 1994. Adenosine: a new agent in the diagnosis of impotence. Int J Impot Res 6:191–198.

Kim Y-C, Zwart M, Chang L, Moro S, Künzel JFD, Melman N, Ijzerman AP, Jacobson KA. 1998. Derivatives of the triazoloquinazoline adenosine antagonist (CGS 15943) having high potency at the human A_{2B} and A_3 receptor subtypes. J Med Chem 41:2835–2845.

Ledent C, Vaugeois J-M, Schiffmann ME, Canderhaeghen J-J, Costentin J, Heath JK, Vassart G, Parmentier M. 1997. Aggressiveness, hyposalgesia and high blood pressure in mice lacking adenosine A_{2A} receptor. Nature 388:674–678.

Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, Rivkees SA, Fink JS, Reppert SM. 1993. Molecular cloning and functional expression of a sheep A3 adenosine receptor with widespread tissue distribution. Mol Pharmacol 44:524–532.

Lupica CR, Cass WA, Zahniser NR, Dunwiddie TV. 1990. Effects of the selective adenosine A2 receptor agonist CGS21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. J Pharmacol Exp Ther 252:1134–1141.

Madara JL, Patapoff TW, Gillece-Castro B, Colgan SP, Parkos CA, Delp C, Mrsny RJ. 1993. 5′-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. J Clin Invest 91:2320–2325.

Mann JS, Holgate ST. 1985. Specific antagonism of adenosine-induced bronchoconstriction is asthma by oral theophylline. Br J Clin Pharmacol 19:685–692.

Marquardt DL, Walker LL. 1990. Modulation of mast cell responses to adenosine by agents that alter protein kinase C activity. Biochem Pharmacol 39:1929–1934.

Marquardt DL, Parker CW, Sullivan TJ. 1978. Potentiation of mast cell mediator release by adenosine. J Immunol 120:871–878.

Marquardt DL, Walker LL, Heinemann S. 1994. Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. J Immunol 152:4508–4515.

Martin PL. 1992. Relative agonist potencies of C2-substituted analogues of adenosine: evidence of adenosine A2b receptors in the guinea pig aorta. Eur J Pharmacol 216:235–242.

Martin PL, Potts AA. 1994. The endothelium of the rat renal artery plays an obligatory role in A2 adenosine receptor-mediated relax-

ation induced by 5′-*N*-ethylcarboxamidoadenosine and N^6-cyclopentyladenosine. J Pharmacol Exp Ther 270:893–899.

Martin PL, Ueeda M, Olsson RA. 1993. 2-Phenylethoxy-9-methyladenine: an adenosine receptor antagonist that discriminates between A2 adenosine receptors in the aorta and the coronary vessels from the guinea pig. J Pharmacol Exp Ther 265:248–253.

Mateo J, Castro E, Zwiller J, Aunis D, Miras-Portugal MT. 1995. 5′-(*N*-ethylcarboxamido)adenosine inhibits Ca^{2+} influx and activates a protein phosphatase in bovine adrenal chromaffin cells. J Neurochem 64:77–84.

Mogul DJ, Adams ME, Fox AP. 1993. Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca^{2+} current in hippocampal CA3 neurons. Neuron 10:327–334.

Moser GH, Schrader J, Deussen A. 1989. Turnover of adenosine in plasma of human and dog blood. Am J Physiol 256:C799–C806.

Murthy KS, McHenry L, Grider JR, Makhlouf GM. 1995. Adenosine A1 and A2b receptors coupled to distinct interactive signaling pathways in intestinal muscle cells. J Pharmacol Exp Ther 274:300–306.

Nakane T, Chiba S. 1990. Adenosine constricts the isolated and perfused monkey coronary artery. Heart Vessels 5:71–75.

Nicholls J, Brownhill VR, Hourani SMO. 1996. Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle muscularis mucosae. Br J Pharmacol 117:170–174.

Nyce JW, Metzger WJ. 1997. DNA antisense therapy for asthma in an animal model. Nature 97:721–725.

Palmer TM, Stiles GL. 1995. Neurotransmitter receptors: VII. Adenosine receptors. Neuropharmacology 34:683–694.

Peachell PT, Columbo M, Kagey-Sobotka A, Lichtenstein LM, Marone G. 1988. Adenosine potentiates mediator release from human lung mast cells. Am Rev Respir Dis 138:1143–1151.

Phillips GD, Rafferty P, Beasley R, Holgate ST. 1987. Effect of oral terfenadine on the bronchoconstrictor response to inhaled histamine and adenosine 5′-monophosphate in nonatopic asthma. Thorax 87:939–945.

Phillips GD, Scott VL, Richards R, Holgate ST. 1989. Effect of nedrocromil sodium and sodium cromoglycate against bronchoconstriction induced by inhaled adenosine 5′-monophosphate. Eur Respir J 2:210–217.

Phillips GD, Ng WH, Church MK, Holgate ST. 1990. The response of plasma histamine to bronchoprovocation with methacholine, adenosine 5′-monophosphate, and allergen in atopic nonasthmatic subjects. Am Rev Respir Dis 90:9–13.

Phillis JW, O'Regan MH, Perkins LM. 1993a. Effect of adenosine receptor agonist on spontaneous and K^+-evoked acetylcholine release from the *in vivo* rat cerebral cortex. Brain Res 605:293–297.

Phillis JW, Perkins LM, O'Regan MH. 1993b. Potassium-evoked efflux of transmitter amino acids and purines from rat cerebral cortex. Brain Res Bull 31:547–552.

Pierce KD, Furlong TJ, Selbie LA, Shine J. 1992. Molecular cloning and expression of an adenosine A2b receptor from human brain. Biochem Biophys Res Commun 187:86–93.

Polosa R, Ng WH, Crimi N, Vanceri C, Holgate ST, Church MK, Mistretta A. 1995. Release of mast cell-derived mediators after endobronchial adenosine challenge in asthma. Am J Respir Crit Care Med 151:624–629.

Prentice DJ, Hourani SMO. 1997. Adenosine analogues relax guinea-pig taenia caeci via an adenosine A_{2B} receptor and a xanthine-resistant site. Eur J Pharmacol 323:103–106.

Rafferty P, Beasley CR, Holgate ST. 1987. The contribution of histamine to immediate bronchoconstriction provoked by inhaled allergen and adenosine-5′monophosphate in atopic asthma. Am Rev Respir Dis 136:369–373.

Ramkumar V, Stiles GL, Beaven MA, Ali H. 1993. The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. J Biol Chem 268:16887–16890.

Rivkees SA, Reppert SM. 1992. RFL9 encodes an A2b-adenosine receptor. Mol Endocrinol 6:1598–1604.

Robeva AS, Woodard RL, Jin X, Gao Z, Bhattacharya S, Taylor HE, Rosin DL, Linden J. 1996. Molecular characterization of recombinant human adenosine receptors. Drug Dev Res 39:243–252.

Rubino A, Ralevic V, Burnstock G. 1995. Contribution of P1-(A2b subtype) and P2-purinoceptors to the control of vascular tone in the rate isolate mesenteric arterial bed. Br J Pharmacol 115:648–652.

Sollevi A, Lagerkranser M, Irestedt L, Gordon E, Lindquist C. 1984. Controlled hypotension with adenosine in cerebral aneurysm surgery. Anesthesiology 61:400–405.

Strohmeier GR, Reppert SM, Lencer WI, Madara JL. 1995. The A2b adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. J Biol Chem 270:2387–2394.

Tofovic SP, Branch KR, Oliver RD, Magee WD, Jackson EK. 1991. Caffeine potentiates vasodilator-induced renin release. J Pharmacol Exp Ther 256:850–860.

Townsend-Nicholson A, Baker E, Sutherland GR, Schofield PR. 1995. Localization of the adenosine A2b receptor subtype gene (ADOARA2B) to chromosome 17p11.2-p12 by FISH and PCR screening. Genomics 25:605–607.

Ukena D, Schirren CG, Schwabe U. 1985. Effects of enprofylline on A1 and A2 adenosine receptors. Eur J Pharmacol 117:25–33.

Ukena D, Jacobson KA, Kirk KL, Daly JW. 1986. A [^{3}H]amine congener of 1,3-dipropyl-8-phenylxanthine: a new radioligand for A2 adenosine receptors of human platelets. FEBS Lett 199:269–274.

van Galen PJM, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, Ijzerman AP, Stiles GP, Jacobson KA. 1994. A binding site model and structure-activity relationships for rat A3 adenosine receptor. Mol Pharmacol 45:1101–1111.

Verani MS, Mahmarian JJ, Hixson JB, Boyce TM, Staudacher RA. 1990. Diagnosis of coronary artery disease by controlled coronary vasodilatation with adenosine and thallium-201 scintigraphy in patients unable to exercise. Circulation 82:80–87.

Vittori S, Camaioni E, Volpini R, Klotz K-N, Cristalli G. 1998. Synthesis and adenosine receptor affinity of (2-Bromo)-9-substituted purines. Drug Dev Res 43:31.

Webb RL, Sills MA, Chovan JP, Balwierczak JL, Francis JE. 1992. CGS 21680: A potent selective adenosine A_2 receptor agonist. Cardiovasc Drug Rev 10:26–53.

Yakel JL, Warren RA, Reppert SM, North RA. 1993. Functional expression of adenosine A2b receptor in *Xenopus* oocytes. Mol Pharmacol 43:277–280.

Zwart M, Vollinga RC, Künzel JFD, Beukers MW, Sleegers D, Ijzerman AP. 1998. Potent antagonists for the adenosine A_{2B} receptor: II. Triazolotriazines with high affinity. Drug Dev Res 43:28.

Adenosine Receptor Activation Induces Vascular Endothelial Growth Factor in Human Retinal Endothelial Cells

Maria B. Grant, Roy W. Tarnuzzer, Sergio Caballero, Mark J. Ozeck, Margaret I. Davis, Polyxenie E. Spoerri, Igor Feoktistov, Italo Biaggioni, John C. Shryock, Luiz Belardinelli

Abstract—Adenosine, released in increased amounts by hypoxic tissues, is thought to be an angiogenic factor that links altered cellular metabolism caused by oxygen deprivation to compensatory angiogenesis. Adenosine interacts with 4 subtypes of G protein–coupled receptors, termed A_1, A_{2A}, A_{2B}, and A_3. We investigated whether adenosine causes proliferation of human retinal endothelial cells (HRECs) and synthesis of vascular endothelial growth factor (VEGF) and, if so, which adenosine receptor subtype mediates these effects. The nonselective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA), in a concentration-dependent manner, increased both VEGF mRNA and protein expression by HRECs, as well as proliferation. This proliferative effect of NECA was inhibited by the addition of anti-human VEGF antibody. NECA also increased insulin-like growth factor-I and basic fibroblast growth factor mRNA expression in a time-dependent manner and cAMP accumulation in these cells. In contrast, neither the A_1 agonist N^6-cyclopentyladenosine nor the A_{2A} agonist 2-*p*-(2-carboxyethyl) phenethylamino-NECA caused any of the above effects of NECA. The effects of NECA were not significantly attenuated by either the A_{2A} antagonist SCH58261 or the A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine. However, the nonselective adenosine receptor antagonist xanthine amine congener completely inhibited the effects of NECA. Addition of antisense oligonucleotide complementary to A_{2B} adenosine receptor mRNA inhibited VEGF protein production by HRECs after NECA stimulation. Thus, the A_{2B} adenosine receptor subtype appears to mediate the actions of adenosine to increase growth factor production, cAMP content, and cell proliferation of HRECs. Adenosine activates the A_{2B} adenosine receptor in HRECs, which may lead to neovascularization by a mechanism involving increased angiogenic growth factor expression. **(*Circ Res.* 1999;85:699-706.)**

Key Words: adenosine receptor ■ angiogenesis ■ ischemia ■ hypoxia ■ diabetes

Tissue hypoxia and ischemia are known to initiate a series of events that lead to the development of collateral blood vessels[1] in a process referred to as compensatory angiogenesis.[2] However, the cellular and molecular mechanisms underlying compensatory angiogenesis have been only partially elucidated.[2] Potential mediators of compensatory angiogenesis include vascular endothelial growth factor (VEGF),[3] basic fibroblast growth factor (bFGF),[4] insulin-like growth factor-I (IGF-I),[5] and nucleosides such as adenosine.[6]

Adenosine, the subject of the present study, has been proposed to be a factor that links altered cellular metabolism caused by oxygen deprivation to the formation of new capillaries.[7–9] This proposed role of adenosine is based on the observation that this nucleoside is released in increased amounts by hypoxic and/or ischemic cells and promotes proliferation of endothelial cells.[9–11] Consistent with this hypothesis, adenosine and adenosine analogs have been reported to affect a number of steps involved in angiogenesis, including endothelial cell proliferation,[8,12–14] migration,[12,15,16] and blood vessel formation in various vascular beds.[15,17] Adenosine can interact with at least 4 subtypes of G protein–coupled receptors, designated A_1, A_{2A}, A_{2B}, and A_3.[18] These receptor subtypes are encoded by distinct genes and can, for the most part, be differentiated on the basis of their affinities for selected agonists and antagonists.[19,20] A_1 and A_3 adenosine receptors are coupled to pertussis toxin–sensitive inhibitory G proteins that inhibit adenylyl cyclase activity, whereas A_{2A} (high-affinity) and A_{2B} (low-affinity) adenosine receptors are coupled to cholera toxin–sensitive G proteins that stimulate adenylyl cyclase activity.[21] In most cell types and organ systems, activation of A_1 adenosine receptors results in decreased work, and therefore, reduced O_2 consumption. Activation of A_{2A} adenosine receptors, on the other hand, increases O_2 supply by causing vasodilation.[22] Thus, adenosine is an ideal metabolite to respond to imbalances between O_2 supply and demand. In the retina, hypoxia is followed by

Received December 22, 1998; accepted August 11, 1999.

From the Departments of Medicine (M.B.G., R.W.T., S.C., M.I.D., P.E.S., J.C.S., L.B.), Ophthalmology (M.B.G.), and Pharmacology and Therapeutics (M.J.O., J.C.S., L.B.), University of Florida, Gainesville, Fla, and Department of Medicine and Pharmacology (I.F., I.B.), Vanderbilt University, Nashville, Tenn. L.B.'s current address is Pharmacological Sciences, CV Therapeutics, Palo Alto, Calif.

Correspondence to M.B. Grant, MD, Associate Professor of Medicine, Department of Medicine, Division of Endocrinology and Metabolism, University of Florida, P.O. Box 100226, Gainesville, FL 32610-0226. E-mail grantma@medicine.ufl.edu

Circulation Research is available at http://www.circresaha.org

compensatory angiogenesis, which is detrimental and results in aberrant blood vessels that are friable and prone to bleeding.[23]

VEGF is a potent endothelial mitogen, induced by hypoxia and hyperglycemia, and has been shown to be an important factor in ischemic ocular neovascularization.[24–26] VEGF causes hyperpermeability of blood vessels, which is observed in both nonproliferative and proliferative diabetic retinopathy. VEGF acts through 2 receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), both of which are tyrosine kinases. VEGF signaling occurs through tyrosine phosphorylation of phospholipase C (PLC) and phosphatidylinositol 3′-kinase.[27,28] The effects of VEGF are also mediated by activation of protein kinase C (PKC) to induce membrane translocation of PKC isoforms, especially the β-isoform of the enzyme.[25] VEGFR-1 mediates the permeability effects associated with VEGF, whereas VEGFR-2 mediates the proliferative effects of VEGF. Elevated levels of VEGF have been detected in vitreous humor of diabetic patients with proliferative retinopathy.[25] More importantly, in animal models of retinal neovascularization, inhibition of VEGF blocks neovascularization.[29] Other growth factors have been implicated in ocular angiogenesis, including bFGF[30] and IGF-I.[31]

The experiments described were performed test the hypothesis that adenosine regulates expression of the angiogenic growth factor VEGF and to determine the adenosine receptor subtype that mediates the effect of the nucleoside in retinal endothelial cells of human origin.

Materials and Methods

5′-*N*-ethylcarboxamidoadenosine (NECA), 2-*p*-(2-carboxyethyl) phenethylamino-NECA (CGS21680), N^6-cyclopentyladenosine (CPA), xanthine amine congener (XAC), and 8-cyclopentyl-1,3-dipropylxanthine (CPX) were from Research Biochemicals, Inc. 5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-E)-1,2,4-trizolo(1,5-*c*) pyrimidine (SCH58261) was a gift from Schering-Plough Research Institute. Rabbit anti-human VEGF was from Peprotech. Antibodies and conjugates for immunofluorescence were as follows. Chicken anti-human A_{2B} adenosine receptor,[32] FITC-conjugated rabbit anti-chicken IgG, and nonimmune sera were from Sigma-Aldrich. Sense and antisense oligonucleotides corresponding to either VEGF or A_{2B} adenosine receptor sequences were synthesized by Life Technologies. The sequences were chosen from the region proximal to and including the start codon using published sequence information for these genes. The antisense sequences are as follows: VEGF, AGACAGCAGAAAGTTCATGG, and A_{2B} adenosine receptor, CAGCGCGTCCTGTGTCTCCAGCAGCATGG. Sense sequences are the complement of the antisense sequences shown.

Human retinal endothelial cells (HRECs) were prepared and maintained as previously described.[33] Cells in passages 3 to 6 were used for the studies. The identity of HRECs was validated by demonstrating endothelial cell incorporation of fluorescently labeled acetylated LDL and by fluorescence-activated cell sorting analysis as previously described.[33] For all experiments, cells were starved of serum overnight and then incubated with adenosine deaminase type III (2 U/mL, Sigma-Aldrich) for 20 minutes before test agents were added. Adenosine receptor agonists and antagonists were added at concentrations ranging from 5 nmol/L up to 100 μmol/L in serum-free medium containing adenosine deaminase type III and then incubated for additional times as indicated in specific results.

cAMP was measured in response to adenosine receptor agonists and/or antagonists as described.[34] Conditioned medium was used to measure changes in VEGF protein in response to adenosine receptor agonists and/or antagonists using an ELISA kit (R&D Systems, Inc). HREC proliferation was determined by measuring DNA synthesis via colorimetric detection of bromodeoxyuridine (BrdU) incorporation using a kit (Roche Molecular Biochemicals), and also by changes in cell number. BrdU incorporation was also used to measure the effect of anti-VEGF antibody on adenosine receptor agonist–induced HREC proliferation. The potential reduction of VEGF synthesis induced by adenosine receptor agonist was tested by ELISA after inclusion of antisense oligonucleotides directed against mRNA for either A_{2B} adenosine receptor or VEGF. Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR), using a competitive synthetic multiplex template as described previously,[35] was performed to measure changes in mRNA after treatment with adenosine receptor agonists and/or antagonists. Immunofluorescent confocal microscopy was used as described[36] to demonstrate both the presence of A_{2B} adenosine receptors and uptake of fluorescence-labeled acetylated LDL in HRECs grown on multichamber glass slides (Nalge Nunc International).

Statistical Analysis

Comparisons between treatment groups (as described in the figure legends) were analyzed by 1-way ANOVA followed by the Bonferroni *t* test. Data are expressed as mean±SEM. Values of $P<0.05$ were considered statistically significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

The nonselective adenosine receptor agonist NECA, after 48 hours of exposure, induced a concentration-dependent increase in DNA synthesis by HRECs, as indicated by BrdU incorporation (Figure 1A). In contrast, neither the A_{2A} adenosine receptor agonist CGS21680 (10 nmol/L to 10 μmol/L) nor the A_1 adenosine receptor agonist CPA (10 nmol/L to 10 μmol/L) increased BrdU incorporation by HRECs (Figure 1A). The addition of the adenosine receptor antagonist XAC completely prevented NECA-stimulated BrdU incorporation (Figure 1B). In contrast, neither the selective A_1 adenosine receptor antagonist CPX (20 nmol/L) nor the selective A_{2A} adenosine receptor antagonist SCH58261 (60 nmol/L) attenuated the stimulatory effect of NECA on BrdU incorporation by HRECs (Figure 1B).

The data for cell counts were consistent with those for BrdU incorporation. Treatment with NECA for 48 hours resulted in a concentration-dependent increase in HREC number, whereas neither CGS21680 nor CPA caused an increase in cell number (Figure 2A). Of the 3 adenosine receptor antagonists tested, only XAC (10 μmol/L) significantly inhibited the increase in cell number induced by 10 μmol/L NECA (Figure 2B).

cAMP Accumulation

To obtain evidence for the presence of the A_{2B} adenosine receptors in HRECs, we performed assays for cAMP content in intact HRECs after treatment of cells with adenosine receptor agonists and antagonists. The nonselective adenosine receptor agonist NECA increased the cAMP content of HRECs in a concentration-dependent manner (Figure 3A), with an EC_{50} value of 24 μmol/L. In contrast, the selective high-affinity A_{2A} adenosine receptor agonist CGS21680 (at concentrations up to 100 μmol/L) had no significant effect on cAMP content of HRECs (Figure 3A). The effect of selective A_1 and A_{2A} adenosine receptor antagonists on NECA-induced accumulation of cAMP was also examined. NECA (10 μmol/L)-

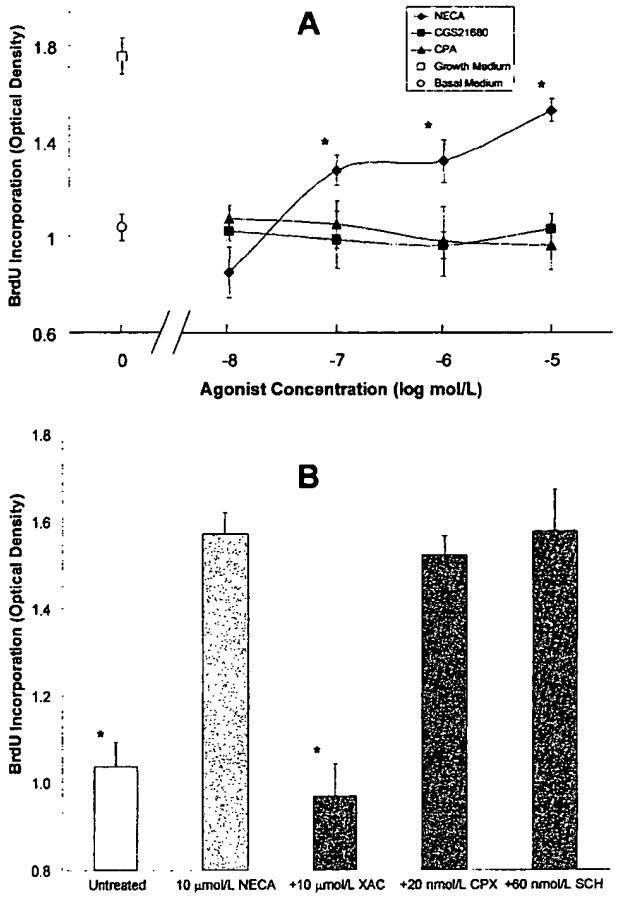


Figure 1. A, DNA synthesis in HRECs exposed to basal medium (○), ie, nutrient medium without supplements; growth medium (□); or basal medium supplemented with increasing concentrations of the selective A_{2A} adenosine receptor agonist CGS21680 (■), the selective A_1 adenosine receptor agonist CPA (▲), or the nonselective adenosine receptor agonist NECA (♦) for 48 hours. DNA synthesis was quantified by measuring BrdU incorporation as described in Materials and Methods. Increase in BrdU incorporation caused by NECA was significantly different ($P<0.05$) from both CGS21680 and CPA at concentrations ranging from 100 nmol/L to 10 μmol/L. *Significantly different from basal medium, by ANOVA ($P<0.05$). B, BrdU incorporation in HRECs exposed simultaneously to the nonspecific adenosine receptor agonist NECA and the selective A_1 receptor antagonist CPX, the selective A_{2A} receptor antagonist SCH58261 (SCH), or the nonselective receptor antagonist XAC for 48 hours. Untreated cells were maintained in basal nutrient medium. *Significantly different from 10 μmol/L NECA, by ANOVA ($P<0.05$). A and B, Data are mean±SEM of 4 independent experiments.

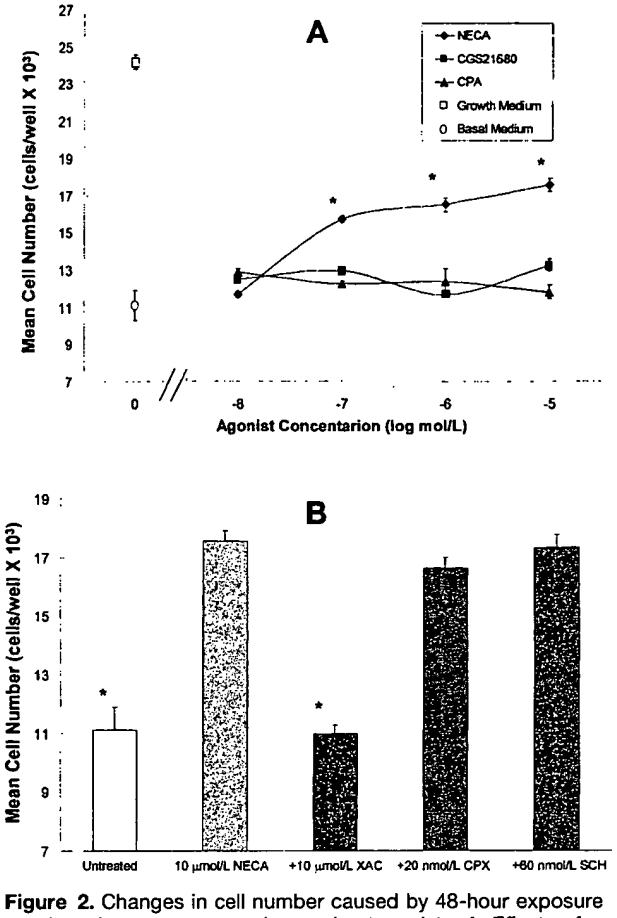


Figure 2. Changes in cell number caused by 48-hour exposure to adenosine receptor agonists and antagonists. A, Effects of NECA (♦), CGS21680 (■), and CPA (▲) on cell number. *Significantly different from basal medium, by ANOVA ($P<0.05$). B, Effects of XAC, CPX, and SCH58261 (SCH) on mean cell number in the presence of 10 μmol/L NECA. *Significantly different from 10 μmol/L NECA, by ANOVA ($P<0.05$). A and B, Data are mean±SEM of 3 different experiments.

induced increase in cAMP content in HRECs was not significantly inhibited either by the selective A_{2A} adenosine receptor antagonist SCH58261 (60 nmol/L) or by the selective A_1 adenosine receptor antagonist CPX (20 nmol/L) (Figure 3B). On the other hand, the nonselective adenosine receptor antagonist XAC (10 μmol/L) completely blocked the effect of NECA on cAMP accumulation.

Quantification of VEGF, IGF-I, and bFGF mRNA in HRECs

To determine whether NECA stimulates angiogenic growth factor mRNA expression in HRECs, total mRNA from HRECs exposed to NECA was subjected to quantitative RT-PCR. Treatment of HRECs with NECA (10 nmol/L to 10 μmol/L) for 2 hours induced a concentration-dependent increase in expression of mRNA for VEGF by up to 4.6-fold (from 0.4×10^6 to 1.85×10^6 copies/μg RNA), compared with untreated control cells. After 8 hours of exposure to NECA, cell mRNA levels for VEGF in cells treated with NECA had returned to baseline. HRECs were also treated with the A_{2A} adenosine receptor agonist, CGS21680 (10 nmol/L to 10 μmol/L), and the A_1 adenosine receptor agonist, CPA (10 nmol/L to 10 μmol/L). In contrast to NECA, neither CGS21680 nor CPA caused a significant change in VEGF mRNA expression (data not shown).

The increase in mRNA for VEGF caused by 10 μmol/L NECA was not attenuated significantly either by the selective A_{2A} adenosine receptor antagonist SCH58261 (60 nmol/L) or by the selective A_1 adenosine receptor antagonist CPX (20 nmol/L) (Figure 4). The nonselective antagonist XAC (10 μmol/L) completely attenuated NECA-induced increases in mRNA for VEGF (Figure 4). NECA (10 μmol/L) also induced a time-dependent increase in mRNA for both IGF-I and bFGF. IGF-I mRNA levels increased by 2.2-fold after 2

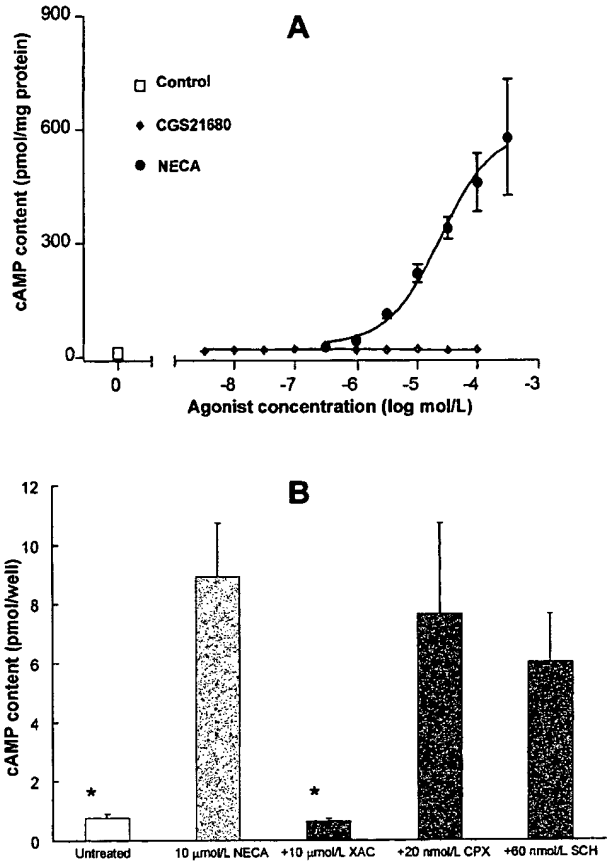


Figure 3. A, cAMP content of HRECs exposed to increasing doses of either the selective A_{2A} adenosine receptor agonist CGS21680 or the nonselective adenosine receptor agonist NECA. Data are mean±SEM of 4 independent experiments. B, cAMP content of HRECs exposed to NECA alone or in combination with either the selective A_1 adenosine receptor antagonist CPX, the selective A_{2A} adenosine receptor antagonist SCH58261 (SCH), or the nonselective adenosine receptor antagonist XAC at the concentrations indicated. Data are mean±SEM of 3 independent experiments. *Significantly different from 10 μmol/L NECA, by ANOVA ($P<0.05$).

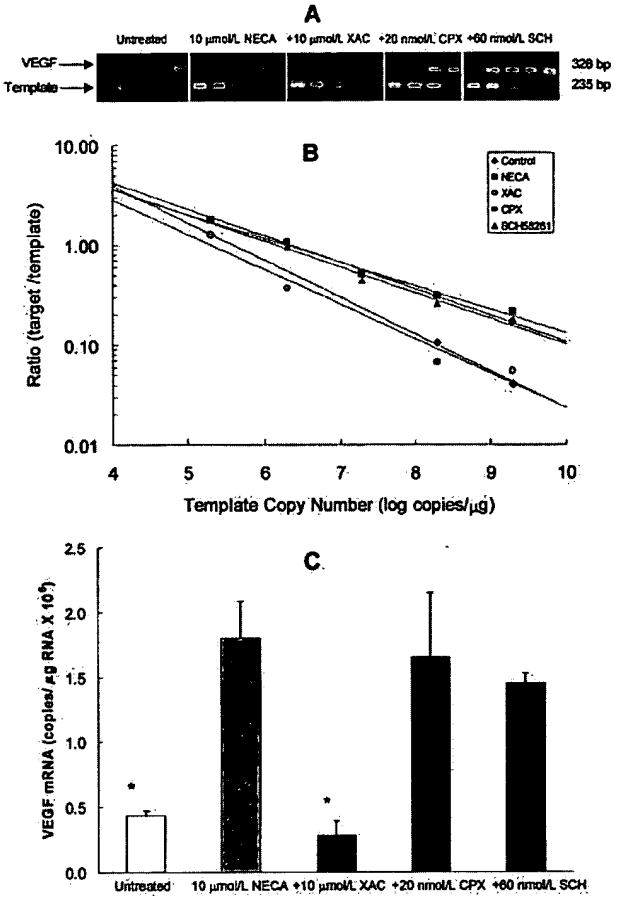


Figure 4. Changes in VEGF mRNA expression in HRECs exposed for 2 hours to the nonselective adenosine receptor agonist NECA alone or in combination with either the selective A_1 adenosine receptor antagonist CPX, the selective A_{2A} adenosine receptor antagonist SCH58261 (SCH), or the nonselective adenosine receptor antagonist XAC at the concentrations indicated. A, Representative photograph of ethidium bromide–stained agarose gel showing the 2 amplification products from the competitive RT-PCR reaction (described in Materials and Methods). The PCR products for the target VEGF and template sequences were 326 and 235 bp, respectively. B, Plot of band intensities of target-to-template ratio vs template copy number. Note the left shift of the line for XAC, indicating a decrease in target mRNA expression to a level similar to that of untreated cells. C, Graphical representation of the data plotted in B. Data are mean±SEM of 2 independent experiments. *Significantly different from 10 μmol/L NECA, by ANOVA ($P<0.05$).

hours of exposure (from 30×10^3 copies/μg RNA to 65×10^3 copies/μg RNA) and 11.7-fold (350×10^3 copies/μg RNA) after 8 hours of exposure to NECA. Similarly, bFGF mRNA increased 3.7-fold after 2 hours of exposure (from 2×10^3 copies/μg RNA to 7.4×10^3 copies/μg RNA) and 11.4-fold (22.8×10^3 copies/μg RNA) after 8 hours of exposure to NECA.

Quantification of VEGF Protein in Conditioned Medium

To determine whether the increase in VEGF mRNA expression resulted in increased protein levels, VEGF was measured in conditioned medium after 8 hours of exposure to NECA, in the presence or absence of adenosine receptor antagonists (Figure 5). NECA increased VEGF protein, but neither the A_1 adenosine receptor agonist CPA (not shown) nor the A_{2A} adenosine receptor agonist CGS21680 caused an increase in VEGF protein (Figure 5). The increase in VEGF protein caused by NECA was not attenuated by either the selective A_{2A} adenosine receptor antagonist SCH58261 (60 nmol/L) or by the selective A_1 adenosine receptor antagonist CPX (20 nmol/L). Only the nonselective adenosine receptor antagonist XAC (10 μmol/L) completely inhibited the action of NECA to increase VEGF protein expression (Figure 5).

Effect of Anti-VEGF Antibody on NECA-Induced HREC Proliferation

Incubation with 10 ng/mL VEGF resulted in BrdU incorporation to a level approximating that induced by normal growth medium. The anti-VEGF antibody at 100 ng/mL significantly reduced DNA synthesis induced by VEGF (Figure 6). Incubation with NECA (10 μmol/L) increased

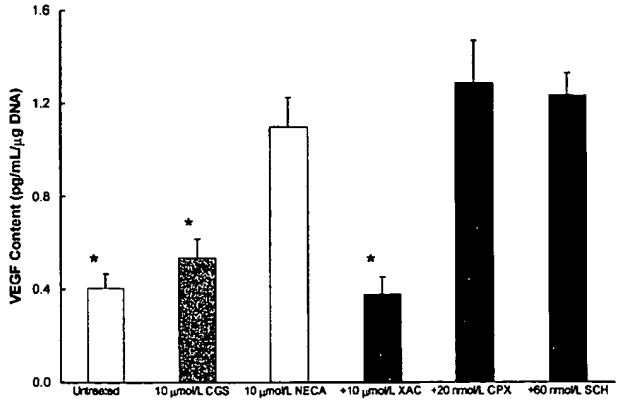


Figure 5. VEGF content in conditioned medium from HRECs exposed for 8 hours to NECA alone or in combination with the selective A_1 adenosine receptor antagonist CPX, the selective A_{2A} adenosine receptor antagonist SCH58261 (SCH), or the nonselective adenosine receptor antagonist XAC at the concentrations indicated. Untreated cells (negative control) and cells exposed to the selective A_{2A} adenosine receptor agonist CGS (agonist control) are shown for comparison. Data are mean±SEM of 4 independent experiments. *Significantly different from 10 μmol/L NECA, by ANOVA ($P<0.05$).

DNA synthesis to levels comparable with that induced by normal growth medium. The addition of anti-VEGF antibody resulted in a decrease in NECA-induced BrdU incorporation, which was statistically significant at the highest concentration of antibody used (Figure 6). Similar results were observed at either 24 or 48 hours of exposure to the test agents.

Effect of Antisense Oligonucleotides on VEGF Induction by NECA

Both A_{2B} adenosine receptor and VEGF antisense oligonucleotides caused a significant decrease of VEGF in the conditioned medium after NECA exposure (Figure 7). This effect was most pronounced for the receptor antisense oligonucleotide with 10 nmol/L NECA, but it was evident for all concentrations of NECA tested. The VEGF antisense oligonucleotide also caused a decrease in secreted VEGF in response to NECA, although not to the same magnitude as that observed with the A_{2B} adenosine receptor antisense.

Immunofluorescence

Analysis of acetylated LDL uptake indicates that the cells are indeed of endothelial origin (Figure 8A). These results were confirmed by immunofluorescent labeling with antibody to coagulation factor VIII (data not shown). Labeling with A_{2B} adenosine receptor antibody clearly demonstrated that the tested cells express the A_{2B} receptor subtype (Figure 8B and 8C).

Discussion

In this report, we demonstrate that the nonselective adenosine receptor agonist NECA, but neither the A_{2A} adenosine receptor agonist CGS21680 nor the A_1 adenosine receptor agonist CPA, stimulates HREC DNA synthesis, proliferation, and cAMP accumulation. Furthermore, neither the selective A_{2A} adenosine receptor antagonist SCH58261 nor the A_1 adenosine receptor selective antagonist CPX attenuated significantly the effects of NECA. Only the nonselective adenosine receptor antagonist XAC reduced significantly the NECA-mediated increase of DNA synthesis, cell proliferation, cAMP content, and VEGF synthesis. The A_{2B} adenosine receptor, which we localized in HRECs using a specific antibody, is the predominant adenosine receptor subtype responsible for mediating the actions of NECA. Equally or more importantly, the addition of VEGF antibody decreased significantly NECA-induced BrdU incorporation. This finding provides strong evidence that VEGF plays a major role in mediating the mitogenic effect of NECA and, presumably, the natural ligand adenosine. We also demonstrated a significant increase in expression of mRNA for both IGF-I and bFGF after 8 hours of exposure to NECA. Thus, our data also raise the possibility that, in addition to VEGF, IGF-I and

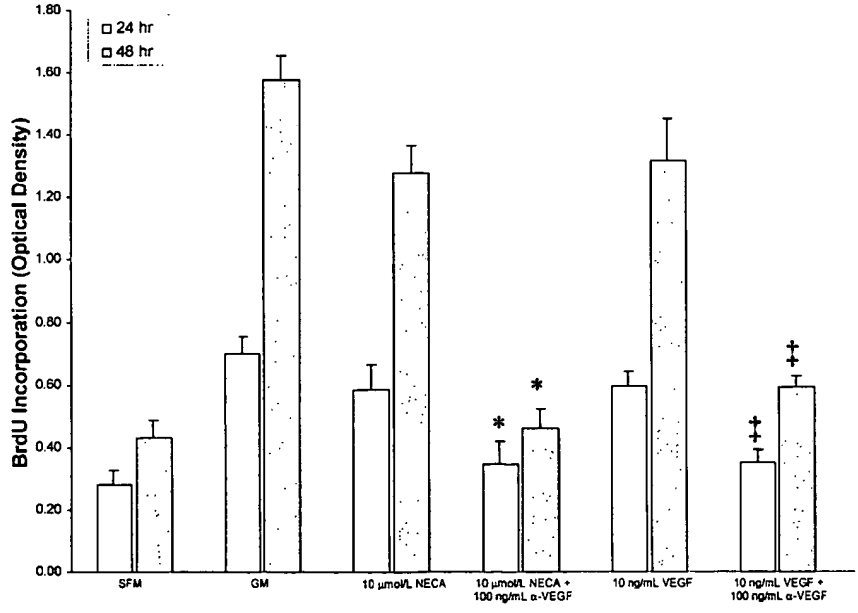


Figure 6. BrdU incorporation as a measure of DNA synthesis in HRECs after stimulation with NECA alone or in combination with a blocking antibody to VEGF. Data are mean±SEM of 3 independent experiments. Open bars are results after 24 hours of exposure; filled bars are results after 48 hours. Serum-free medium (SFM) and normal growth medium (GM) were used as negative and positive controls, respectively. *Significantly different from 10 μmol/L NECA alone for the respective exposure time, by ANOVA ($P<0.05$). ‡Significantly different from 10 ng/mL VEGF alone for the respective exposure time, by ANOVA ($P<0.05$).

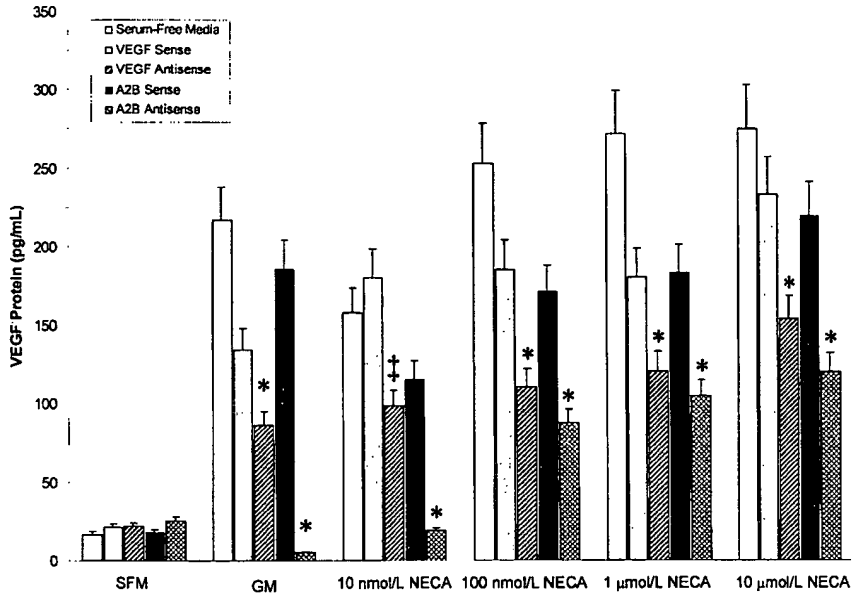


Figure 7. VEGF content in conditioned medium from HRECs after stimulation with NECA in the presence or absence of sense or antisense oligonucleotides homologous to the human A_{2B} adenosine receptor or to human VEGF. Assay duration was 48 hours. The presence of sense oligonucleotide serves to control for nonspecific oligonucleotide effects. Data are mean±SEM of 3 determinations. *Significantly different from the respective condition (GM or increasing concentrations of NECA) receiving no oligonucleotide, by ANOVA ($P<0.01$). ‡Significantly different from 10 μmol/L NECA, by ANOVA ($P<0.05$).

bFGF may contribute in part to the proliferative effect of NECA.

In HRECs, NECA caused a concentration-dependent increase in VEGF mRNA as well as an increase in secreted VEGF protein that was blocked by an antisense oligonucleotide complementary to A_{2B} adenosine receptor mRNA. In contrast, neither the A_{2A} agonist CGS21680 nor the A_1 agonist CPA affected the expression either of VEGF mRNA or protein, ruling out a role for either A_{2A} or A_1 adenosine receptors in mediating increased VEGF expression, increased BrdU incorporation, and cell proliferation. NECA-induced increases in expression of both VEGF mRNA and protein by HRECs were blocked by the nonselective adenosine receptor antagonist XAC, whereas the A_1- and A_{2A}-selective adenosine receptor antagonists CPX (20 nmol/L) and SCH58261 (60 nmol/L), respectively, did not attenuate these increases. The antagonists CPX and SCH58261 were used at concentrations at which their selectivity for A_1 and A_{2A} receptors has been demonstrated in cardiovascular preparations.[18,37,38] On the other hand, XAC was used at a concentration (10 μmol/L) that should be sufficient to antagonize effectively A_1, A_{2A}, A_{2B}, and possibly the A_3 receptor-mediated responses.

Hence, the evidence supporting the role of A_{2B} adenosine receptors as the adenosine receptor subtype that mediates the effects of NECA reported here can be summarized as follows: (1) A_{2B} receptors were localized in HRECs using immunofluorescence microscopy with the A_{2B} antibody; (2) antisense oligonucleotides homologous to the A_{2B} receptor blocked NECA-stimulated VEGF production; (3) neither the A_1 nor the A_{2A} receptor agonists had any effect on BrdU incorporation, cell proliferation, or cAMP production; (4) neither the A_1 nor the A_{2A} antagonists, used at the concentration at which they are selective for their receptor subtype, antagonized the effects of NECA; and (5) the nonselective but potent A_{2B} antagonist XAC used at high concentrations significantly attenuated the effects of NECA.

Taken together, the data support the hypothesis that the A_{2B} adenosine receptor, but neither the A_1 nor the A_{2A} receptor, is responsible for mediating the actions of NECA on cAMP accumulation and VEGF synthesis in cultured HRECs. The results of our studies do not rule out a possible role of A_3 receptor in mediating the effects of NECA. However, this is unlikely, because the affinity (K_i) of XAC for the A_3 adenosine receptor is 29 μmol/L, which is higher than the concentration (10 μmol/L) used in our studies.[37,39] Our conclusion that the A_{2B} receptor is the most likely adenosine receptor subtype that mediates the effects of NECA—and presumably adenosine—on HRECs differs from that reported by Takagi et al.[40,41] These investigators, using retinal endothelial cells of bovine origin, concluded that the proliferative action of adenosine is mediated by A_{2A} receptor. Takagi et al[40] also reported that acute hypoxia causes a decline in KDR/Flk mRNA levels as well as VEGF binding sites on the cell surface. On the other hand, chronic hypoxia was associated with increased KDR/Flk message levels.[40] More importantly, Takagi et al[41] also reported that the endogenous adenosine released by hypoxic bovine retinal endothelial cells was sufficient to stimulate VEGF message expression. Species differences and passage number may account for differences in the observed adenosine receptor subtype in retinal endothelial cells. Furthermore, distinct adenosine receptor subtypes may mediate the proliferative effects of adenosine in endothelial cells from different vascular beds, even within the same species.

Protein kinase A and members of the mitogen-activated protein kinase, family such as extracellular signal–regulated kinases 1 and 2 (ERK1/ERK2) are potential mediators of adenosine–mediated cell proliferation.[42–44] Activation of the A_{2B} adenosine receptor results in cAMP generation via G_s. A_2 receptor activation and stimulation of adenylyl cyclase/protein kinase A pathways can either activate[11] or inhibit[45–47] growth factor–stimulated ERK activity.

A_{2B} adenosine receptor signaling through $G_{q/11}$ also results in increased levels of ERK.[42] Therefore, A_{2B} receptor stimulation of $G_{q/11}$, PLC, and PKC may synergize with or potentiate the effects of traditional tyrosine kinase–

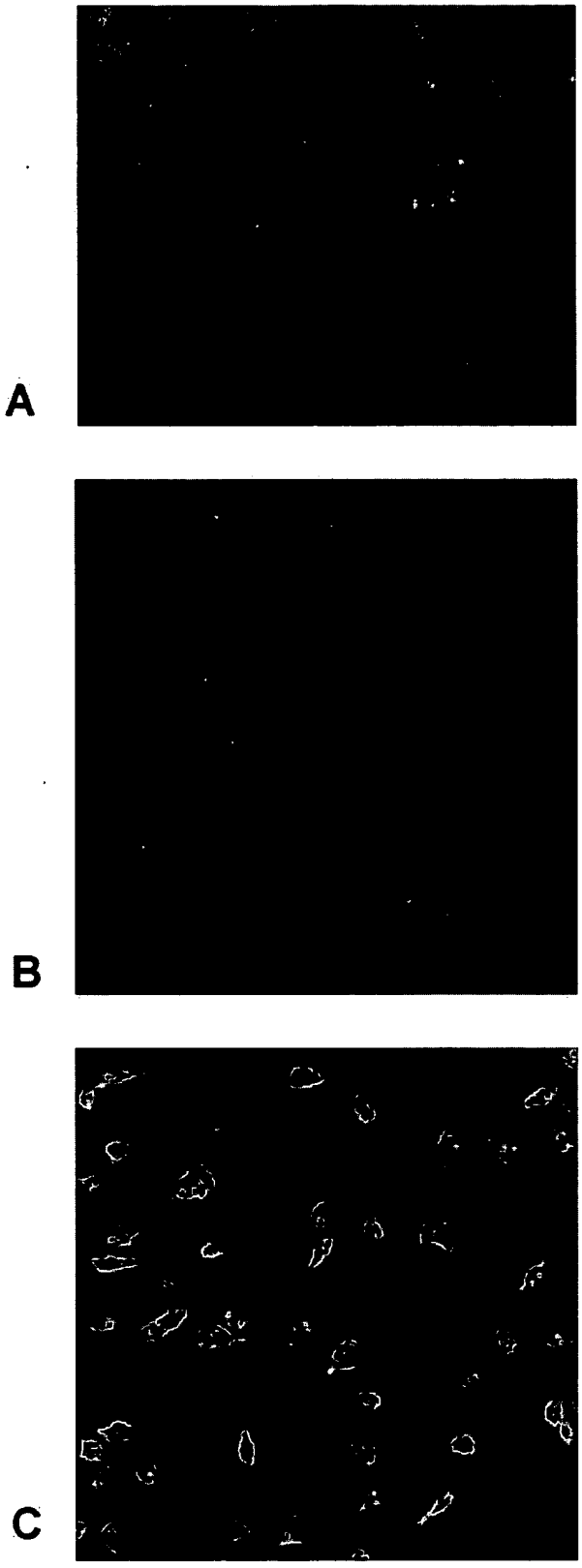


Figure 8. A, Fluoromicrograph of subconfluent HRECs that were incubated for 6 hours with 5 μg/mL fluorescence-labeled acetylated LDL. Magnification, ×120. Cells were visualized using a standard FITC long-pass excitation:emission filter set. Note the abundance of punctate perinuclear fluorescence in the HRECs. Cultured human coronary artery smooth muscle cells were used as a negative control and showed no evidence of acetylated LDL uptake (data not shown). B, Confocal image of HRECs at ≈50% confluence reacted with FITC-conjugated rabbit anti-chicken Ab showing nonspecific binding of this antibody. Magnification, ×60. C, Confocal image of HRECs at ≈50% confluence reacted first with AF5 chicken anti-A_{2B} adenosine receptor Ab, followed by FITC-conjugated rabbit anti-chicken Ab, demonstrating specific reactivity of the cells to the anti-A_{2B} receptor antibody. Magnification, ×60.

coupled growth factors,[48,49] either through c-*src*–dependent activation of ERK or through PKC-dependent, *src*-independent, pathways. Because forskolin-mediated adenylyl cyclase activity does not activate VEGF expression in endothelial cells,[50] $G_{q/11}$- and PKC-mediated activation of ERK may contribute to activation of transcription factors and lead to the induction of message for VEGF.[51] Thus, A_{2B} receptor activation can mediate proliferation by inducing growth factor synthesis and through stimulation of $G_{q/11}$, PLC, and PKC pathways.

Angiogenesis is a compensatory mechanism in response to insufficient tissue oxygenation.[2] In the retina of diabetic individuals, homeostatic abnormalities lead to retinal nonperfusion and subsequent ischemia.[52] Ischemia leads to new vessel formation and disruption of the normal retinal vasculature, the hallmarks of proliferative diabetic retinopathy.[23] Our findings raise the possibility that selective A_{2B} adenosine receptor antagonists could be used as a novel therapeutic approach to block the inciting events leading to aberrant angiogenesis in proliferative diabetic retinopathy. Pharmacological modulation of the neovascular response in a nondestructive manner should have significant advantages over current therapeutic approaches.

By blocking the A_{2B} adenosine receptor, the action of adenosine to induce the growth factor cascade may be inhibited, and blocking the A_{2B} adenosine receptor may attenuate aberrant cellular proliferation. In summary, our results provide strong evidence that the proliferative effect of adenosine on HRECs is caused by increased expression of VEGF and probably other growth factors, and this effect is mediated by the A_{2B} adenosine receptor.

Acknowledgments

We thank Dr Donald Szarowski (Wadsworth Center, 3-D Light Microscopy Facility, Albany, NY) for assistance with confocal imaging.

References

1. Folkman J, Shing Y. Angiogenesis. *J Biol Chem.* 1992;267:10931–10934.
2. Dor Y, Eli K. Ischemia-driven angiogenesis. *Trends Cardiovasc Med.* 1997; 7:289–294.
3. Ferrara N, Davis Smith T. The biology of vascular endothelial growth factor. *Endocr Rev.* 1997;18:4–25.
4. Gospodarowicz D, Bialecki H, Thakral TK. The angiogenic activity of the fibroblast and epidermal growth factor. *Exp Eye Res.* 1979;28:501–514.
5. Grant MB. Insulinlike growth factor-I in diabetic vascular complications. *Curr Opin Endocr Diabetes.* 1996;3:335–345.
6. Dusseau J, Hutchins P. Hypoxia-induced angiogenesis in chick chorioallantoic membranes: a role for adenosine. *Respir Physiol.* 1988;17:33–44.
7. Roth T, Podesta F, Stepp MA, Boeri D, Lorenzi M. Integrin overexpression induced by high glucose and by human diabetes: potential pathway to cell dysfunction in diabetic microangiopathy. *Proc Natl Acad Sci USA.* 1993; 90:9640–9644.

8. Dusseau J, Hutchins P, Malbasa D. Stimulation of angiogenesis by adenosine on the chick chorioallantoic membrane. *Circ Res.* 1986;59:163–170.
9. Meininger CJ, Granger HJ. Mechanisms leading to adenosine-stimulated proliferation of microvascular endothelial cells. *Am J Physiol.* 1990;258: H198–H206.
10. Nees S, Herzog V, Becker BF, Bock M, Des Rosiers CH, Gerlach E. The coronary endothelium: a highly active metabolic barrier for adenosine. *Basic Res Cardiol.* 1985;80:515–529.
11. Sexl V, Mancusi G, Holler C, Gloria Maercker E, Schutz W, Freissmuth M. Stimulation of the mitogen-activated protein kinase via the A2A-adenosine receptor in primary human endothelial cells. *J Biol Chem.* 1997;272: 5792–5799.
12. Meininger CJ, Schelling ME, Granger HJ. Adenosine and hypoxia stimulate proliferation and migration of endothelial cells. *Am J Physiol.* 1988;255: H554–H562.
13. Sexl V, Mancusi G, Baumgartner Parzer S, Schutz W, Freissmuth M. Stimulation of human umbilical vein endothelial cell proliferation by A2-adenosine and beta 2-adrenoceptors. *Br J Pharmacol.* 1995;114:1577–1586.
14. Ethier MF, Chander V, Dobson JG Jr. Adenosine stimulates proliferation of human endothelial cells in culture. *Am J Physiol.* 1993;265:H131–H138.
15. Hudlicka O, Wright A, Ziada A. Angiogenesis in the heart and skeletal muscle. *Can J Cardiol.* 1986;2:120–123.
16. Van Daele P, Van Coevorden A, Roger PP, Boeynaems JM. Effects of adenine nucleotides on the proliferation of aortic endothelial cells. *Circ Res.* 1992;70:82–90.
17. Adair T, Montani J, Strick D, Guyton A. Vascular development in chick embryos: a possible role for adenosine. *Am J Physiol.* 1989;256:H240–H246.
18. Shryock J, Belardinelli L. Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. *Am J Cardiol.* 1997;79:2–10.
19. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M. Nomenclature and classification of purinoceptors. *Pharmacol Rev.* 1994;46:143–156.
20. Collis M, Hourani S. Adenosine receptor subtypes. *Trends Pharmacol Sci.* 1993;14:360–366.
21. Stiles G. Adenosine receptors. *J Biol Chem.* 1992;267:6451.
22. Belardinelli L, Shryock J. Does adenosine function as a retaliatory metabolite in the heart? *News Physiol Sci.* 1992;7:52–56.
23. Frank RN. On the pathogenesis of diabetic retinopathy: a 1990 update. *Ophthalmology.* 1991;98:586–593.
24. Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. *Am J Pathol.* 1994;145:574–584.
25. Aiello L. Vascular endothelial growth factor: 20th-century mechanisms, 21st-century therapies. *Invest Ophthalmol Vis Sci.* 1997;38:1647–1652.
26. Kirkpatrick CJ, Wagner M, Hermanns I, Klein CL, Kohler H, Otto M, van Kooten TG, Bittinger F. Physiology and cell biology of the endothelium: a dynamic interface for cell communication. *Int J Microcirc Clin Exp.* 1997; 17:231–240.
27. Mukhopadhyay D, Nagy J, Manseau E, Dvorak H. Vascular permeability factor/vascular endothelial growth factor-mediated signaling in mouse mesentery vascular endothelium. *Cancer Res.* 1998;58:1278–1284.
28. Gerber H, McMurtrey A, Kowalski J, Yan M, Keyt B, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway: requirement for flk-1/kdr activation. *J Biol Chem.* 1998;273:30336–30343.
29. Miller J, Adamis A, Aiello L. Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. *Diabetes Metab Rev.* 1997;13:37–50.
30. Hanneken A, Lutty GA, McLeod DS, Robey F, Harvey AK, Hjelmeland LM. Localization of basic fibroblast growth factor to the developing capillaries of the bovine retina. *J Cell Physiol.* 1989;138:115–120.
31. Grant MB, King GL. IGF-1 and blood vessels. *Diabetes Rev.* 1995;3: 113–128.
32. Feoktistov I, Sheller J, Vallejo V, Biaggioni I. Immunological identification of adenosine A2B receptors in human lung mast cells. *Drug Dev Res.* 1996;37:146–150.
33. Grant MG, Guay C. Plasminogen activator production by human retinal endothelial cells of nondiabetic and diabetic origin. *Invest Ophthalmol Vis Sci.* 1991;32:53–64.
34. Shryock JC, Ozeck MJ, Belardinelli L. Inverse agonists and neutral antagonists of recombinant human A1 adenosine receptors stably expressed in Chinese hamster ovary cells. *Mol Pharmacol.* 1998;53:886–893.
35. Tarnuzzer RW, Macauley SP, Farmerie WG, Caballero S, Ghassemifar MR, Anderson JT, Robinson CP, Grant MB, Humphreys-Beher MG, Franzen L, Peck AB, Schultz GS. Competitive RNA templates for detection and quantitation of growth factors, cytokines, extracellular matrix components and matrix metalloproteinases by RT-PCR. *Biotechniques.* 1996;20:670–674.
36. Davis-Cox MI, Turner JN, Szarowski D, Shain W. Phorbol ester-stimulated stellation in primary cultures of astrocytes from different brain regions. *Microsc Res Tech.* 1994;29:319–327.
37. Shryock J, Snowdy S, Baraldi P, Cacciari B, Spalluto G, Monopoli A, Ongini E, Baker S, Belardinelli L. A2A adenosine receptor reserve for coronary vasodilation. *Circulation.* 1998;98:711–718.
38. Belardinelli L, Shryock J, Snowdy S, Zhang Y, Monopoli A, Lozza G, Ongini E, Olsson R, Dennis D. The A2A adenosine receptor mediates coronary vasodilation. *J Pharmacol Exp Ther.* 1998;284:1066–1073.
39. Daly J, Jacobson K. Adenosine receptors: selective agonists and antagonists. In: Belardinelli L, Pelleg A, eds. *Adenosine and Adenine Nucleotides: From Molecular Biology to Integrative Physiology.* Boston, Mass: Kluwer Academic Publishers; 1995:157–166.
40. Takagi H, King G, Ferrara N, Aiello L. Hypoxia regulates vascular endothelial growth factor receptor KDR/Flk gene expression through adenosine A2 receptors in retinal capillary endothelial cells. *Invest Ophthalmol Vis Sci.* 1996;37:1311–1321.
41. Takagi H, King GL, Robinson GS, Ferrara N, Aiello LP. Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells. *Invest Ophthalmol Vis Sci.* 1996;37:2165–2176.
42. Feoktistov I, Goldstein AE, Biaggioni I. Role of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase kinase in adenosine A2B receptor-mediated interleukin-8 production in human mast cells. *Mol Pharmacol.* 1999;55:726–734.
43. Grant MB, Tarnuzzer RW, Davis MI, Caballero S, Spoerri PE, Ozeck MJ, Shryock JC, Belardinelli L. Adenosine activates mitogen activated protein kinase (MAPK) and angiogenic growth factor production in human retinal endothelial cells (HRECs). In: The Endocrine Society 8th Annual Meeting, June 27, 1998; New Orleans, La. Bethesda, Md: The Endocrine Society; 1998. Abstract.
44. Grant MB, Davis MI, Tarnuzzer RW, Caballero S, Belardinelli L. Adenosine mediates human retinal endothelial cell (HREC) chemotaxis, capillary tube formation and stimulatory signaling cascades. *Invest Ophthalmol Vis Sci.* 1999;40:S616.
45. D'Angelo G, Lee H, Weiner RI. cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. *J Cell Biochem.* 1997;67:353–366.
46. Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. *Science.* 1993;262:1065–1069.
47. Cook SJ, McCormick F. Inhibition by cAMP of Ras-dependent activation of Raf. *Science.* 1993;262:1069–1072.
48. Ray LB, Sturgill TW. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. *Proc Natl Acad Sci USA.* 1988;85:3753–3757.
49. Schonwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol.* 1998;18:790–798.
50. Pueyo ME, Chen Y, D'Angelo G, Michael JB. Regulation of vascular endothelial growth factor expression by cAMP in rat aortic smooth muscle cells. *Exp Cell Res.* 1998;238:354–358.
51. Milanini J, Vinals F, Pouyssegur J, Pages G. p42/p44 MAP kinase module plays a key role in the transcriptional regulation of the vascular endothelial growth factor gene in fibroblasts. *J Biol Chem.* 1998;273:18165–18172.
52. Alexander SP, Cooper J, Shine J, Hill SJ. Characterization of the human brain putative A2B adenosine receptor expressed in Chinese hamster ovary (CHO.A2B4) cells. *Br J Pharmacol.* 1996;119:1286–1290.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 5, Issue of February 3, pp. 2387–2394, 1995
Printed in U.S.A.

The A_{2b} Adenosine Receptor Mediates cAMP Responses to Adenosine Receptor Agonists in Human Intestinal Epithelia*

(Received for publication, August 15, 1994, and in revised form, November 15, 1994)

Gregg R. Strohmeier‡§, Steven M. Reppert¶, Wayne I. Lencer‖, and James L. Madara‡

From the ‡Division of Gastrointestinal Pathology, Department of Pathology, Brigham and Women's Hospital, the ¶Department of Pediatrics, Massachusetts General Hospital and ‖Children's Hospital, and the Departments of Pathology and Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Adenosine is thought to be a major effector in immunological stimulation of Cl^- secretion in intestinal epithelia. Previous studies indicate that both apical and basolateral domains of intestinal epithelial cells possess functionally defined adenosine receptors. However, it is unclear whether the same receptor subclass is expressed, what the receptor subclass(es) is, or how the receptors signal the Cl^- secretory response. We now characterize the intestinal epithelial adenosine receptor subtype using the model epithelium, T84. Both apical and basolateral adenosine receptor agonist response profiles revealed a hierarchy (ED_{50}) of 5′-(*N*-ethylcarboxamido)adenosine > adenosine > CGS-21680. Similarly, inhibition studies revealed identical ID_{50} hierarchies for apical and basolateral antagonism by xanthine amine congener > 1,3-diethyl-8-phenylxanthine > aminophylline. Analyses of both agonist and antagonist pharmacological hierarchies in Chinese hamster ovary cells stably expressing the A_{2b} receptor revealed these same hierarchies. Northern blots performed on RNA extracted from polarized T84 monolayers demonstrated no detectable message for A_1 or A_{2a} adenosine receptor, but strong hybridization was detected for the A_{2b} adenosine receptor. Subsequent Northern blots of RNA prepared from human alimentary tract revealed that A_{2b} adenosine receptor message was heavily expressed throughout the colon, in the appendix, and more modestly expressed in the small intestine (ileum). Analyses of cAMP generation in T84 cells in response to adenosine indicated that the basolateral A_{2b} receptor elicits Cl^- secretion through this signaling pathway. Stimulation of Cl^- secretion through the apical A_{2b} receptor exhibited relatively small but significant increases in cAMP compared with basolateral stimulation. The protein kinase A inhibitor H-89, used at concentrations that did not affect short circuit current responses to the Ca^{2+}-mediated agonist carbachol, effectively inhibited short circuit current elicited by either apical or basolateral adenosine. These data suggest that the major intestinal epithelial adenosine receptor is the A_{2b} subclass, which is positively coupled to adenylate cyclase. Such observations have potentially important implications for the treatment of diarrheal diseases.

The purine nucleoside adenosine regulates ion transport in a variety of epithelia. For example, adenosine elicits electrogenic Cl^- secretion in a variety of epithelia (1–3). In intestinal epithelia, this Cl^- secretory pathway results in movement of isotonic fluid into the lumen, a process that naturally serves to hydrate the mucosal surface but, in the extreme, produces secretory diarrhea (4, 5). Up-regulation of this secretory mucosal flush often parallels active inflammatory responses elicited by lumenal pathogens and, by reducing the duration of colonization by these pathogens, serves as a crude form of mucosal defense (4, 6, 7). We have recently shown that both polymorphonuclear leukocytes (PMN)[1] and eosinophils, when activated, release a soluble agonist that directly stimulates electrogenic Cl^- secretion by intestinal epithelial cells (8–10). Subsequent studies have shown that this PMN-eosinophil-derived secretagogue is 5′-AMP, which is rapidly converted to adenosine at the epithelial surface via the ectoenzyme, ecto-5′-nucleotidase (11). In addition to release of 5′-AMP by PMN and eosinophils, release of adenosine by mast cells is also thought to be a key mediator of mucosal inflammation (1, 12, 13). These data support the notion that adenosine serves as a major direct-acting secretagogue in a variety of inflammatory states.

Studies performed using intestinal mucosal sheets or cultured human intestinal epithelial cells such as T84 cells, which appropriately model Cl^- secretion, indicate that adenosine is an effective secretagogue whether placed apically or basolaterally (11, 14). The presence of apical receptors on these polarized epithelial cells is conceptually important. Inflammatory cells (PMN and eosinophils) respond to lumenal pathogens by migrating across the epithelium (7, 15, 16). Once in the lumen, paracrine release of 5′-AMP permits efficient conversion to adenosine, since the glycosylphosphatidylinositol-linked ectoenzyme is expressed in polarized fashion on the apical membrane of enterocytes (11). Thus, engagement of the apical 5′-AMP-adenosine signaling pathway subsequent to transepithelial migration facilitates coupling of two defenses: (i) translocation of inflammatory cells into the threatened lumenal compartment and (ii) subsequent volume flush of the mucosal surface. However, it is unclear which adenosine receptor subclass(es) are expressed by intestinal epithelial cells, whether apical and basolateral membranes express different receptor subtypes and how the specific subclass of adenosine receptor(s) expressed are coupled to signaling cascades that permit activation of electrogenic Cl^- secretion. Several subclasses of adenosine receptors (AdoR) exist (A_1, A_{2a}, A_{2b}, A_3), all of which are

* This work was supported by National Institutes of Health Grants DK-35932, DK-47662, DK-42125, and DK-48106. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Pathology, Brigham and Women's Hospital, 20 Shattuck St., Thorn 1419, Boston, MA 02115. Tel.: 617-732-6528; Fax: 617-732-6796.

[1] The abbreviations used are: PMN, polymorphonuclear leukocytes; NECA, 5′-(*N*-ethylcarboxamido)adenosine; XAC, xanthine amine congener; DPX, 1,3-diethyl-8-phenylxanthine; AM, aminophylline; DPM, dipyridamole; CHO cells, Chinese hamster ovary cells; AdoR, adenosine receptor; HBSS, Hanks' balanced salt solution; Isc, short circuit current; IBMX, 3-isobutyl-1-methylxanthine; PCR, polymerase chain reaction.

examples of guanine nucleotide-coupled receptors (17–23). Each AdoR subclass appears to exhibit restricted tissue expression and unique pharmacokinetics (17–19, 24). Studies using mammalian expression systems suggest that all of these adenosine receptors may couple to adenylate cyclase (17–21, 23, 24). In contrast, studies of Ado-elicited intestinal Cl^- secretion indicated that the AdoR signaling pathway in this epithelium does not appear to involve a cAMP, cGMP, or an intracellular calcium signal (8, 11, 25, 26).

Here, we report that the polarized human intestinal epithelial cell line, T84, which serves as a predictive and widely used model for the study of regulated intestinal Cl^- secretion, exclusively expresses the recently cloned A_{2b} adenosine receptor subclass. The basolateral A_{2b} receptor is positively coupled to adenylate cyclase as assessed by measurement of intracellular cAMP. Signaling through the apical receptor by adenosine also elicits a dose-dependent increase in intracellular cAMP, which is significantly smaller than that observed in cells stimulated with basolateral adenosine. However, specific inhibition of protein kinase A confirms that both apical and basolateral adenosine receptor signal transduction is mediated by cAMP. Lastly, analyses of A_{2b} message in mucosal samples from along the human alimentary tract suggest that the A_{2b} receptor is richly expressed at many sites, particularly in the colon. These data may have important implications for structuring new therapies for diarrheal disease associated with intestinal inflammation in humans.

MATERIALS AND METHODS

Cell Culture—Approximately 10^3 monolayers were used for these studies. Confluent monolayers of the human intestinal epithelial cell line T84 were grown on collagen-coated permeable supports and maintained until steady-state resistance was achieved, as previously described (27). The configuration of the majority of monolayers used was identical to that previously developed for a microassay (28). Measurements of transepithelial resistance, voltage, and short-circuit current (Isc) were performed in Hanks' balanced salt solution (HBSS) using standard biophysical techniques as previously described (9, 27, 28). Studies of cAMP generation were performed both using 0.33- and 5-cm^2 inserts. Chinese hamster ovary cells (CHO) transfected with the mouse A_{2b} receptor were grown in 24-well plates in the presence of positive selection (geneticin) as described (19). For assays of Ado responses, CHO cells were washed and refed with media lacking geneticin 2 days prior to use, grown 2 days, and then washed with HBSS. Responses to adenosine analogs were analyzed as described below.

cAMP Measurement—Measurements of cAMP were performed on ethanol extracts of cells obtained from monolayers grown on permeable supports, using a radioimmunoassay kit as directed by the supplier (DuPont NEN). Briefly, after monolayers were washed with $HBSS^+$ and incubated 10 min, base line Isc readings were taken, and then agonists were added by the addition of 10% volume buffer containing agonist. For antagonist studies, the added 10% volume contained both adenosine and specific antagonist. The dose-response curves for antagonist inhibition of adenosine cAMP responses were performed using 100 μM adenosine (a dose at which cAMP increases 1 order of magnitude above base line). 5 min after stimulation, short circuit responses were acquired to verify the level of Cl^- secretion. At 10 min, reservoirs were aspirated from the monolayers, and 4 °C $HBSS^+$ was added to stop the reaction. Quickly, the filters on which monolayers rested were cut from the plastic supports and placed in eppendorfs containing extract buffer (66% ethanol, 33% $HBSS^+$) at 4 °C. Where indicated, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (1 mM, Sigma) was included in the extract buffer. Monolayers were compacted and centrifuged, and an aliquot (100 μl) was withdrawn for the radioimmunoassay.

Molecular Analyses—Northern blot analysis was performed on poly $(A)^+$ RNA isolated from six 5-cm^2 monolayers of T84 cells carefully scraped off the filters in cold HBSS and concentrated by low speed centrifugation. Primary tissue was acquired from the frozen section room of Brigham and Women's Hospital, mucosa stripped of the underlying muscularis, rapidly frozen on aluminum foil set on dry ice, and stored until use. Total RNA was isolated using the guanidium-thiocyanate method, and poly$(A)^+$ RNA was isolated using oligo(dT) (29). 5 μg of poly$(A)^+$ RNA were fractionated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane (GeneScreen, DuPont NEN). Blots were hybridized with the requisite AdoR cDNA ^{32}P-labeled by the method of random priming (specific activity > 10^9 cpm/μg). Hybridization reactions were performed in 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and denatured salmon sperm (100 μg/ml) at 42 °C overnight. The final wash of blots was 0.2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 1% SDS at 65 °C for 40 min. Blots were exposed to x-ray film with an intensifying screen at −80 °C for 3 days.

Reverse Transcriptase-PCR Probing for AdoR Subclass Expression—Poly $(A)^+$ RNA was prepared from T84 cells using established methods. 2 μg of the RNA were primed with oligo(dT) and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega). The first strand cDNA was subjected to two rounds of 30 cycles each of PCR amplification with 1 μg of primer A (TCAGAATTCTA(T/C)ATGGTITACT(A/T)(C/T)AA(C/T)TT(C/T)TT) and primer B (TTCAAGCTTGGIA(A/G)CCA(A/G)(C/G)(A/T)IA(A/G)IGC(A/G)AA). Each reaction cycle consisted of incubations at 94 °C for 1.5 min, 45 °C for 2 min, and 72 °C for 2 min with Ampli *Taq* DNA polymerase (Perkin-Elmer Corp.). The amplified DNA was digested with *Hin*dIII and *Eco*RI and separated on an agarose gel. A prominent DNA band of approximately 220 base pairs was apparent, which was subsequently used to prepare and sequence recombinant clones as previously described (19).

Reagents—All tissue culture supplies were obtained from Life Technologies, Inc., and cAMP radioimmunoassay kits were from DuPont NEN. AdoR agonists and antagonists and Ado uptake inhibitors were purchased from Research Biochemicals Inc. (Natick, MA), except adenosine. H-89 was obtained from Seikagaku America, Inc. (Rockville, MD). All other reagents were obtained from Sigma and Calbiochem.

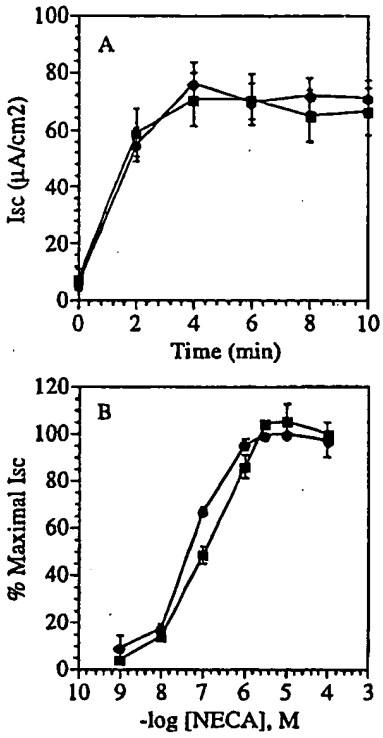


FIG. 1. **Isc time course and dose response of T84 cells to NECA.** Both panels display the T84 Isc response to apical (■) or basolateral (●) stimulation with 1 μM NECA. The *upper panel* (*A*) illustrates the time course of T84 Isc response to NECA, while the *bottom panel* (*B*) represents the dose response. Each *point* represents the mean of at least four experiments performed in duplicate or triplicate.

RESULTS

Adenosine Receptor Stimulation—A Isc representing electrogenic Cl^- secretion may be elicited from T84 cells by the application of adenosine or its analogs to either the apical or basolateral membrane (11, 13, 14). Fig. 1 shows that both apical and basolateral NECA, a non-metabolizable AdoR agonist, rapidly stimulate a Isc response from T84 cells (dIsc/dt; peak Isc =

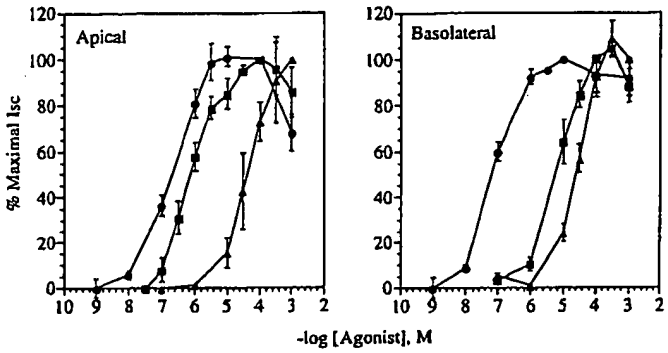


FIG. 2. **Isc dose-response curves for adenosine agonists.** Apical (*left panel*) and basolateral (*right panel*) Isc stimulation of T84 cells by NECA (●), Ado (■), and CGS-21680 (▲) were performed in duplicate, with each *point* representing the mean of at least four experiments. Data are presented as the percent of the maximal Isc response observed in each experiment.

70.75 ± 8.85 $\mu A/cm^2$ for apical and 69.92 ± 6.17 $\mu A/cm^2$ for basolateral stimulation). The NECA-elicited increases in Isc for apical and basolateral stimulation show nearly identical time constants (apical 25.53 $\mu A/cm^2$/min and basolateral 24.56 $\mu A/cm^2$/min) and dose dependence (ED_{50} = 0.16 ± 0.03 μM and 0.06 ± 0.02 μM, respectively) (Fig. 1*B*). The T84 monolayers used for these studies had high electrical resistance (800–1200 ohm·cm^2), as is typical for this cell line (27). Such severe restriction on the passive permeation of small hydrophilic solutes permits sidedness of responses to be clearly separated since agonists will not diffuse across the monolayer to activate receptors on the opposing membrane. Thus, these data confirm the presence of adenosine receptors on both apical and basolateral membranes and suggest that there is little difference in signaling from apical or basolateral membrane receptors when stimulated by NECA.

T84 AdoR Subclass (Pharmacological Properties)—We next used pharmacologic approaches to characterize the properties of apically and basolaterally expressed adenosine receptors. In Fig. 2, the T84 Isc dose responses elicited by three AdoR agonists are shown. Either apical or basolateral stimulation with Ado, CGS-21680, or NECA induced a dose-dependent Isc response in T84 cells, with the hierarchy for the ED_{50} doses being identical for both apical and basolateral stimulation (NECA > Ado > CGS-21680, Fig. 2 and Table I). The dose dependences for NECA-induced secretion were identical for apical and basolateral receptors, and the secretory responses to four other agonists revealed only modest differences between ED_{50} values for apical *versus* basolateral stimulation (Table I). However, T84 cells were 10-fold more sensitive to authentic adenosine applied to apical rather than basolateral cell surfaces (Fig. 2, *A* and *B*, respectively; apical 0.63 ± 0.16 μM ED_{50} *versus* basolateral 7.78 ± 1.77 μM ED_{50}). This difference in Ado-elicited Isc appears attributable to Ado uptake by the basolateral membrane, resulting in a shift of the dose-response curve to the left. Inhibition of the basolateral Ado uptake process by dipyridamole (DPM), 4-nitrobenzyl-6-thioguanosine, or 4-nitrobenzyl-6-thioinosine all shifted the basolateral Ado dose curve to the left (Fig. 3). The ED_{50} values of the resulting Ado dose-response curves in the presence of these inhibitors was 1 μM, 1.6 μM, and 1 μM, respectively, values approximating that observed for apical stimulation. Since one of the inhibitors of Ado uptake, DPM, may also exhibit phosphodiesterase inhibitory effects, IBMX was used as a control to show that the DPM effect on the dose-response curve could not be explained by phosphodiesterase inhibition. None of these inhibitors altered the apical Ado Isc response (not shown). Lastly, the apical Ado dose-response curve and the leftward shifted basolateral Ado + Ado uptake inhibitor dose-response curves not only overlay each other but are highly similar to the Ado dose-response curves measured in CHO cells transfected with the same Ado receptor subtype, which leads to the T84 cell responses (see below). Thus, such data indicate that the relative insensitivity of the basolateral response to Ado is due to depletion of the basolateral microenvironment due to the activity of basolaterally polarized adenosine uptake.

The AdoR antagonist inhibition profiles (inhibition of response elicited by the Ado ED_{50} dose for the apical or basolateral stimulation, 1 and 10 μM, respectively) are shown in Fig. 4 and summarized in Table I. Again, both the apical and basolateral receptors demonstrated identical antagonist hierarchies (XAC > DPX > AM).

The above data suggested that the same receptor subclass was expressed apically and basolaterally and, by comparison to published antagonist/agonist hierarchies (17–19), that the A_{2b} receptor, recently cloned from rat brain and highly expressed in rat colon, might underlie these responses. Thus, we analyzed agonist/antagonist hierarchies in CHO cells stably transfected with the A_{2b} AdoR and compared the results with those obtained from T84 cells. Responses in CHO cells were measured as cAMP generation since it has previously been shown that this receptor is positively coupled to adenylate cyclase. As shown in Fig. 5, the pharmacology of the expressed A_{2b} receptor in CHO cells mirrored that of the apical and basolateral receptors of T84 cells (NECA > Ado > CGS-21680 for agonist ED_{50} values and XAC > DPX > AM for antagonist ID_{50} values).

AdoR Subclass mRNA in T84 and Natural Human Intestine—To verify that A_{2b} receptors are expressed on T84 cells, we next performed Northern blots using specific cDNA probes for the A_1, A_{2a}, and A_{2b} AdoR. As shown in Fig. 6, only A_{2b}-specific cDNA hybridized with mRNA from T84 cells, revealing two transcripts of approximately 2.4 and 1.7 kilobases. To examine whether additional AdoR family members may be expressed in T84 cells, reverse transcriptase-PCR was used employing a set of degenerate oligonucleotides. Primers were based on regions of the fifth and sixth transmembrane domains that are conserved among the A_1, A_{2a}, and A_{2b} AdoR cDNAs. Fragments of each of the three AdoR cDNAs were amplified. No other AdoR-like fragments were present, indicating the absence of expression of novel adenosine receptors that share the general homologies of this receptor family (data not shown).

We next determined whether the A_{2b} receptor was expressed in the human intestine. Previous *in situ* hybridization studies of rat intestine have suggested that the epithelium represents the major site within this tissue in which the AdoR is expressed.[2] As shown in Fig. 7, the A_{2b} receptor appeared to be expressed at many levels of the human alimentary tract including the esophagus, gastric antrum, weakly in the small intestine, and heavily throughout the colon. Again, two transcripts comparable in size with those noted in T84 cells were present.

Ado- and Ado Analog-mediated cAMP Generation in T84 Cells—We next examined whether the A_{2b} receptor was positively coupled to adenylate cyclase in the highly polarized T84 cell, as it is in the unpolarized CHO cell (18). As shown in Fig. 8, all three agonists studied (Ado, CGS-21680, and NECA) elicited a cAMP response in T84 cells both apically (Fig. 8*A*) and basolaterally (Fig. 8*B*). The rank order of potency for the agonist cAMP ED_{50} values was the same as that observed for the Isc response, that is, NECA > Ado > CGS-21680 (see also Table I), both apically and basolaterally. Strikingly, however, the ability to generate cAMP in response to apical *versus* basolateral agonists differed. As seen in Fig. 8, basolateral ago-

[2] S. A. Rivkees and S. M. Reppert, unpublished observations.

TABLE I

ED_{50} of adenosine receptor agonists and antagonists

Isc was assessed after agonist addition to either the apical or basolateral reservoir, with data acquired every 2 min. The 6-min time point (steady-state) was graphed; the average of duplicate Isc data points were converted to percent of the maximal response and plotted against agonist dose. From this graph, the dose at which the half-maximal response occurred was determined. The mean of the average ED_{50} values was determined from multiple experiments and is reported in μM, with standard error of the means (n = 4 Ado, 7 CGS-21680, and 10 NECA; n = 3 for all other experiments). For antagonist studies, the drugs were suspended in HBSS$^+$ containing an ED_{50} dose of Ado (1 μM for apical stimulation and 10 μM for basolateral), and the data for the ID_{50} was calculated as in the agonist experiments, with n = 3.

Agonist	Isc		Antagonist	Isc	
	Apical	Basolateral		Apical	Basolateral
	ED_{50}, μM			ID_{50}, μM	
Ado	0.63 ± 0.16	7.78 ± 1.77	AM	23.00 ± 1.23	14.33 ± 4.26
CGS-21680	36.86 ± 9.61	18.22 ± 2.04	DPX	3.97 ± 0.91	2.33 ± 0.74
NECA	0.16 ± 0.03	0.06 ± 0.02	XAC	0.03 ± 0.01	0.06 ± 0.02
2-CADO	3.67 ± 0.83	1.27 ± 0.50	8PT	0.26 ± 0.13	0.29 ± 0.16
R-PIA	3.09 ± 0.60	2.40 ± 0.50			
S-PIA	15.00 ± 2.45	11.43 ± 1.47			

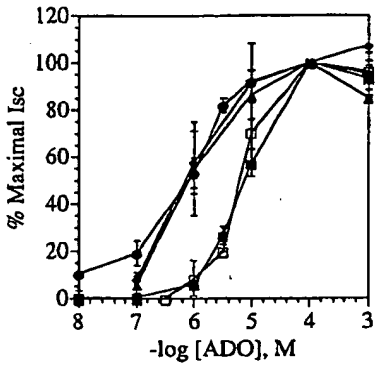


FIG. 3. **Isc response in the presence of adenosine uptake inhibitors.** Basolateral adenosine (■) was added to T84 cells previously incubated with the Ado uptake inhibitors 4-nitrobenzyl-6-thioguanosine (▲) or 4-nitrobenzyl-6-thioinosine (♦), or with the phosphodiesterase inhibitor IBMX (□) or with DPM (●), which possesses both Ado uptake and phosphodiesterase inhibitory activity. All inhibitors were diluted 10× in HBSS$^+$ and 10% volume added to the cells to initiate incubation. Data are the average of two experiments performed in duplicate.

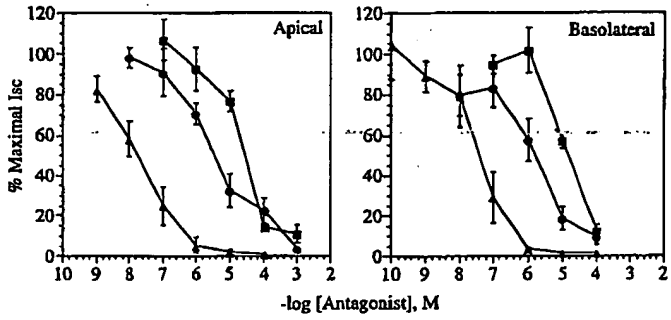


FIG. 4. **Isc dose-response curves for adenosine antagonists added apically or basolaterally to T84 cells.** Apical (*left panel*) and basolateral (*right panel*) antagonists were dissolved in HBSS$^+$ buffer containing an ~ED_{50} dose of adenosine (1 μM apical or 10 μM basolateral final Ado concentrations) and added to cells, and Isc responses were acquired. Each *point* represents the mean of at least three separate experiments performed in duplicate. The antagonists used were XAC (▲), DPX (●), and AM (■). Data are presented as percent of the Isc response to Ado alone for that experiment.

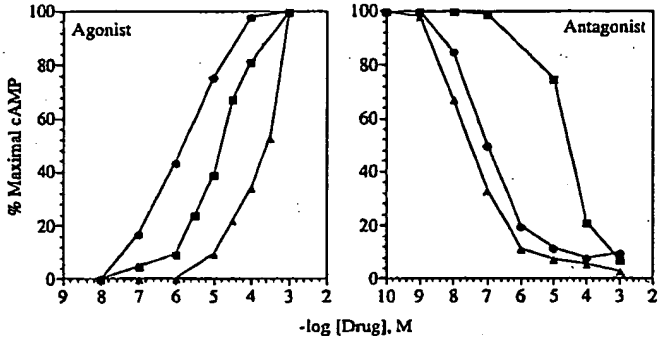


FIG. 5. **cAMP dose-response curves for Ado agonists and antagonists from CHO cells stably transfected with the A_{2b} AdoR.** The *left panel* illustrates the agonist stimulation of CHO cells by NECA (●), Ado (■), and CGS-21680 (▲) added to cells in HBSS$^+$ buffer, while the *right panel* shows the antagonist inhibition of CHO cell cAMP responses elicited by 10 μM Ado (antagonists dissolved in HBSS$^+$ containing Ado and then added to cells). Antagonists used were XAC (▲), DPX (●), and AM (■). In both *panels*, each *point* represents the mean of three separate experiments performed in duplicate. Data are presented as percent of the maximal cAMP response observed in each experiment.

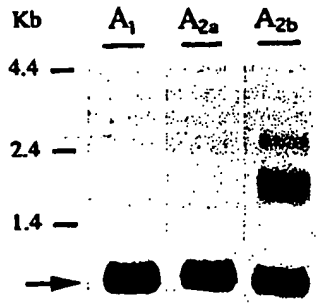


FIG. 6. **Expression of adenosine receptor subclass in T84 cells.** Poly(A)$^+$ RNA (20 μg) isolated from T84 cells was hybridized with specific ^{32}P-labeled cDNA probes for the A1, A2a, and A2b adenosine receptors. *Dark bands* represent specific hybridization signals, with a myosin loading control designated by the *arrow*. RNA size markers (Life Technologies, Inc.) are shown in the *left column*. *Kb*, kilobases.

nists were able to generate cAMP signals in 10–30-fold excess of the signals generated by apically applied agonists. The pharmacology of antagonist inhibition of the basolateral Ado-elicited cAMP responses was also examined. Fig. 9 shows that the antagonist ID_{50} profile in T84 cells stimulated with 100 μM Ado added basolaterally, a dose chosen for its large cAMP increase in the control cells, had a pharmacological hierarchy of XAC > DPX > AM. The order of the antagonist inhibition is identical to that shown for inhibition of Isc response in T84 cells (Fig. 4, Table I) and inhibition of cAMP generation in transfected CHO cells (Fig. 5).

Relationship between A_{2b}-generated cAMP and Isc Responses—It has been previously suggested that Ado analog-mediated Cl^- secretion in T84 cells is not linked to cAMP generation (8, 11, 26). Thus, we defined the relationship between Ado and adenosine analog-mediated cAMP generation and the Isc response. A key aspect in considering such relation-

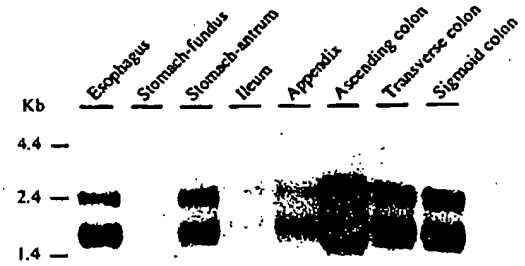


FIG. 7. **Northern blot analysis of the A_{2b} adenosine receptor expression in eight different human tissues.** Poly(A)$^+$ RNA (20 μg) isolated from rapidly frozen natural human tissues acquired through surgery was hybridized with a ^{32}P-labeled A2b adenosine receptor cDNA probe. *Dark bands* represent specific hybridization signals. RNA size markers (Life Technologies, Inc.) are shown in the *left column*. *Kb*, kilobases.

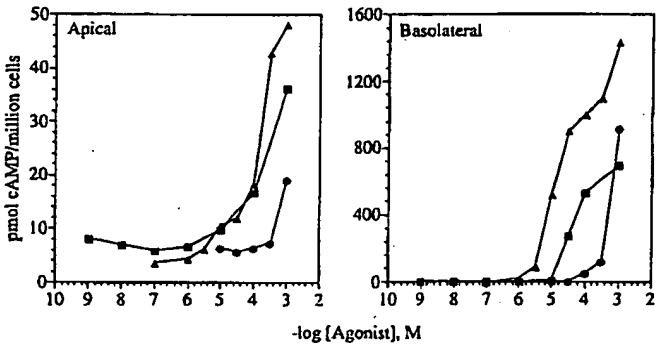


FIG. 8. **cAMP dose-response curves for adenosine agonist stimulation in T84 cells.** Agonists were added apically (*left panel*) or basolaterally (*right panel*), the reaction was allowed to run 10 min, and then cells were lysed and the generated cAMP concentrations were analyzed. Each *point* represents the mean of at least three separate experiments performed in duplicate. Agonists used were NECA (●), Ado (■), and CGS-21680 (▲). Data are presented as percent of the Isc response to Ado alone for that experiment.

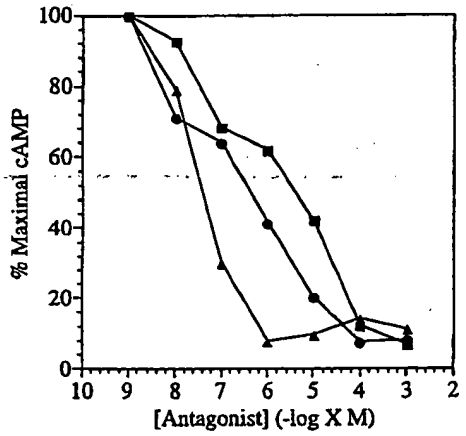


FIG. 9. **cAMP dose-response curves for adenosine antagonist inhibition of basolateral Ado stimulation in T84 cells.** Antagonists were dissolved in HBSS$^+$ buffer containing 100 μM Ado (final concentration), the response was allowed to proceed for 10 min; then cells were lysed, and the generated cAMP concentrations were analyzed. Each *point* represents the mean of at least three separate experiments performed in duplicate. Antagonists used were XAC (▲), DPX ( ), and AM (■). Data are presented as percent of the Isc response to Ado alone for that experiment.

ships, as pointed out by comparing the data in Figs. 2 and 8, is that the ability of T84 cells to secrete Cl^-, as manifested by Isc, saturates well before the ability of the cell to generate cAMP. Therefore, using forskolin as a positive control for cAMP-mediated Isc, we focused on the physiologically relevant (for

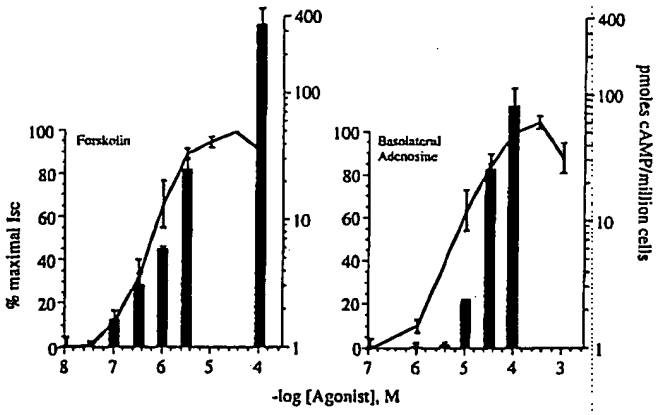


FIG. 10. **Correlation between T84 cAMP and Isc responses to forskolin or adenosine stimulation.** Basolateral stimulation of T84 cells was elicited by forskolin (*left panel*) or adenosine (*right panel*), and the Isc (*curves*) and cAMP (*bars*) responses were analyzed. All *points* were performed in duplicate or triplicate using IBMX in the ethanol extract buffer and represent the responses observed in four separate experiments. Data are presented as the mean percent of the maximal Isc response observed in each experiment on the *left axes*, and the picomoles of cAMP-generated/monolayer of T84 cells are indicated in a representative experiment on the *right axes*.

Isc) dose range and found that detectable and graded increments in cAMP occur with ascent through the Isc dose-response curve (Fig. 10). However, the cAMP response continues to rise beyond the point where the Isc response is saturated. Such subsequent rises in cAMP generation, which are non-physiological with regard to the already maximal Isc response, dwarf the cAMP responses that occur in the agonist concentration range for stimulated Isc (Fig. 10*A*). This point is readily apparent by comparing the cAMP responses from Fig. 8*B* (which approach 10^3 pmol cAMP/10^6 cells after basolateral exposure to agonists) with those from Fig. 10*B* (which, in the range of the Isc response, are less than 10^2 pmol cAMP/10^6 cells). In response to basolateral adenosine, a graded increase in cAMP was observed within the range of the doses required to elicit a secretory response. Moreover, the quantity of cAMP generated in response to agonist concentrations within the dose-response curve was similar between forskolin and basolateral adenosine. These data strongly suggest that the basolateral A_{2b} receptor is positively linked to adenylate cyclase. Ligand binding to the basolateral receptor then elicits a Isc response via cAMP.

In contrast, cAMP generation following apical exposure to Ado was less clearly related to the Isc response (Fig. 11*B*). While significant and progressive increases in cAMP generation could be measured in the Ado concentration range of 3×10^{-6} to 10^{-4} M (roughly corresponding to the range of ED_{70} saturation), no significant increase in cAMP generation was measurable at the Ado concentration corresponding to the ED_{50}. In addition, the cAMP responses observed for the dose range corresponding to ED_{75}–ED_{100} were small (<10%) compared with those observed in this same area of the dose-response curves for forskolin or basolateral Ado (Figs. 10 and 11). Therefore, to further test whether the cAMP-dependent protein kinase mediates Isc in response to both apical and basolateral adenosine stimulation, the Isc responses to Ado, forskolin, or carbachol were assessed in the presence of the protein kinase A-specific antagonist H-89. As shown in Fig. 12, the cAMP-mediated response to forskolin and both the apical or basolateral responses to Ado were comparably inhibited by increasing doses of H-89. In contrast, Isc responses to the calcium-mediated agonist carbachol were unaffected by the identical doses of H-89. These results, coupled with the data

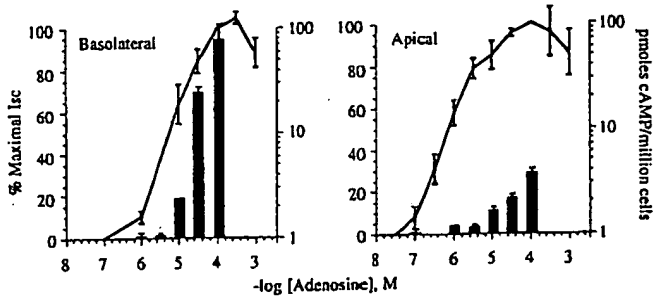


FIG. 11. **Comparison between apical and basolateral Ado stimulation of T84 cAMP and Isc responses.** Adenosine stimulation of T84 cells was elicited basolaterally (*left panel*) or apically (*right panel*), and the Isc (*curves*) and cAMP (*bars*) responses were analyzed. All *points* were performed in duplicate using IBMX in the ethanol extract buffer and represent the responses observed in four separate experiments. Data are presented as the mean percent of the maximal Isc response observed in each experiment on the *left axes*, and the picomoles of cAMP-generated/monolayer of T84 cells are indicated in a representative experiment on the *right axes*.

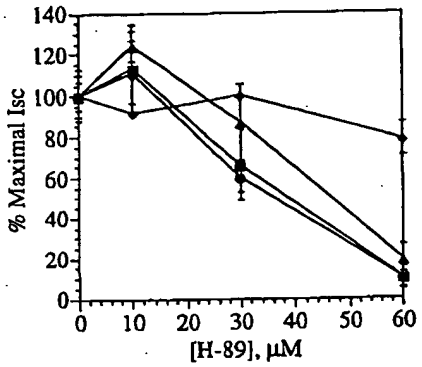


FIG. 12. **Inhibition of cAMP-dependent protein kinase.** Adenosine stimulation of T84 cells was elicited apically (■) or basolaterally ( ) with adenosine or by forskolin (▲) or carbachol (◆), and the Isc responses were analyzed in the presence of increasing doses of protein kinase A-specific inhibitor H-89. All *points* are the average of two experiments performed in duplicate and are presented as the mean percent of the control Isc response observed in each experiment on the *left axes*.

presented above relating the Isc and cAMP dose curves for Ado, strongly suggest that both apical and the basolateral AdoR-mediated Isc responses signal via the cAMP-protein kinase A pathway.

DISCUSSION

These studies show that intestinal epithelial cells, as modeled by the human cell line T84, express both apical and basolateral AdoRs that pharmacologically behave as A_{2b} adenosine receptors. Northern blots of T84 mRNA using subclass-specific probes reveal strong expression of the A_{2b} AdoR but no detectable expression of the A_1 or A_{2a} subclasses of receptor. Furthermore, reverse transcriptase-PCR did not identify any *new* AdoR subtypes in T84 cells. The strong expression of the A_{2b} receptor by natural colonic tissue confirms that the A_{2b} subclass of AdoR is expressed in the natural tissue as well as the widely utilized T84 model. Lastly, we show that adenosine signals Cl^- secretion via cAMP in T84 cells.

Signaling of Cl^- Secretion through the A_{2b} AdoR—Previous studies of adenosine-elicited Cl^- secretion in T84 cells (elicited by apical (neutrophil-derived secretagogue (8)) or basolateral + apical (Ado and analogs (26)) stimulation) have suggested that this receptor does not signal through cAMP. For example, others have found that the dose-response curve of cAMP generation, elicited by the Ado analog NECA, is shifted 1 order of magnitude to the right of that for Isc generation (26). Two pieces of information from the current study help explain this. First, the cAMP responses to basolateral addition of adenosine over the physiologically relevant dose range are small compared to those obtained at doses above saturation. To observe such small changes, a phosphodiesterase inhibitor was needed in the original lysis buffer (data not shown). Second and more importantly, however, it appears that the ability of T84 cells to generate cAMP far exceeds the concentration of cAMP needed to saturate the Cl^- secretory response. Thus, even with the known cAMP-mediated Cl^- secretagogue forskolin (4), the cAMP signals generated throughout the Cl^- secretory dose-response curve are dwarfed by those that follow saturation of the secretory response, thus shifting the ED_{50} by at least a magnitude. Since it appeared that the size of the Ado-elicited cAMP signal throughout the secretory dose-response curve is comparable with that throughout the forskolin dose-response curve, the response elicited by the basolateral A_{2b} receptor is well accounted for by an A_{2b} adenylate cyclase-cAMP signaling pathway. This fits well with reports that the physiological response profile, based on the additive and/or potentiating interactions of adenosine analogs with other secretagogues, was identical to that expected for a cAMP-based response (8, 26).

In contrast to the basolateral receptor, the signaling pathway linking the apical A_{2b} receptor to the Cl^- secretory response seemed less straightforward initially. In the presence of phosphodiesterase inhibitors in the lysis buffer and throughout the cAMP assay, minor but significant increments in cAMP were observed within the Isc dose-response curve following apical stimulation (Fig. 11). However, such increments are not apparent until the ED_{50} dose for Isc generation has been exceeded. On the surface, these data appear to negate a positive coupling of apical A_{2b} with adenylate cyclase. However, the dose-dependent increase in cAMP elicited by apically applied Ado and the closely correlated pharmacology between apical agonist stimulation of Isc and cAMP indicated a relationship between Isc and cAMP. Since the Isc dose-response curve is shifted one log to the left for apical *versus* basolateral stimulation (see below), with the apical response occurring rapidly, and since T84 monolayers severely restrict apical to basolateral diffusion of solutes, the small cAMP increments seen with addition of apical Ado at concentrations of 5–10 μM can almost certainly not be attributed to Ado leak to the basolateral compartment with subsequent binding to the basolateral receptor. Therefore, we further investigated the relationship between cAMP and Isc in Ado-stimulated T84 cells using a cAMP-dependent protein kinase inhibitor, H-89. While H-89 comparably inhibited forskolin, apical Ado, and basolateral Ado-elicited Isc responses over the same dose range, these concentrations of the inhibitor had little or no effect upon the carbachol-stimulated Isc. These data strongly suggest that, like A_{2b} receptors in CHO cells and basolateral A_{2b} receptors in T84 cells, the apical A_{2b} receptor is also coupled to adenylate cyclase. If so, these data imply that T84 cells express a small pool of adenylate cyclase on the apical membrane in close proximity to apically localized cAMP-dependent Cl^- channels. Thus, signal transduction via apical AdoRs may occur at low receptor occupancy (ED_{50} > 10-fold lower) via efficient coupling to adenylate cyclase, protein kinase A, and Cl^- conductance pathways (30–32). Such efficiency would simply reflect the close spatial relation between the components of this putative signal-transducing pathway.

Given these data above, it seems that explanations for apical signaling by adenosine via a cAMP-protein kinase A-independent pathway may not be necessary. Such alternative explanations would have included the possibility that the A_{2b} receptor may be linked directly to Cl^- channels via heterotrimeric

GTPases. The serpentine receptor family to which adenosine receptors belong are, as a paradigm, linked heterotrimeric G-proteins (24). Since the apical receptor resides in the same membrane domain as the regulated Cl^- channel and since it has been shown that G-proteins can directly regulate the cAMP-responsive Cl^- channel, CFTR (33), it is possible that subclasses of receptors within this family could via a direct cAMP-independent pathway regulate Cl^- secretion. Additionally, Barrett and Bigby (34) have recently reported evidence of phospholipid remodeling and arachidonic acid release as a consequence of exposure of T84 cells to adenosine analogs. These observations raise the possibility that signal transduction following apical stimulation might be influenced by a lipid-derived mediator. Finally, the possibility exists that multiple signaling pathways might be involved in mediating the apical Ado response, thus permitting potentiation of responses as observed in other cell types and systems (35–38). While the present study clearly indicates that cAMP-protein kinase A signaling is crucial to the Isc response to signaled by apical adenosine, the possibility that such small cAMP responses interact synergystically with other mediator pathways (such as those possibilities outlined above) are not ruled out by our findings.

Polarization of the Secretory Response Elicited by the Natural Ligand—Using the adenosine analog NECA, others have previously shown that the Isc dose response was not significantly different between apical and basolateral stimulation, although it was noted that the size of the response to basolateral stimulation was modestly greater than that for apical stimulation (14). We previously reported that a buffer conditioned by a neutrophil-derived secretagogue preferentially elicited Cl^- secretion from the apical membrane, with little evidence of basolateral secretory activity observable (8, 39). When this neutrophil-derived agonist was later defined as 5'-AMP, it was also recognized that the concentrations present in solutions conditioned by activated neutrophils were in the low μM range. As shown here, adenosine in this concentration range is only an effective secretagogue when applied apically. Indeed, the ED_{50} for adenosine is 0.63 ± 0.16 μM apically but 7.78 ± 1.77 μM basolaterally. In contrast, the basolateral ED_{50} values for five other non-metabolized Ado analogs were 30–50% of those for apical stimulation. Such data imply a slightly greater sensitivity to basolateral as compared with apical stimulation for metabolically stable AdoR agonists. Thus, it is likely the greater apical sensitivity for the natural agonist reflects the presence of a catabolic pathway restricted to the basolateral domain. Such "catabolism" represents uptake of basolateral adenosine, a mechanism identified in other cell types (1, 2), since inhibition of Ado uptake by three inhibitors shifted the basolateral adenosine dose curve to the left, closely approximating the apical dose curve. In addition, we have found that neither the adenosine deaminase inhibitor deoxycoformycin nor the 5'-nucleotidase inhibitor α,β-methylene-ADP alters the ED_{50} shift observed between apical and basolateral adenosine stimulation (not shown).

T84 Cells and Natural Human Intestine Express the A_{2b} AdoR (Therapeutic Implications)—Stehle *et al.* (18) recently used PCR-based approaches to clone a novel adenosine receptor subtype from rodent brain, which they classified as the A_{2b} receptor based on its ligand binding characteristics and its apparent positive coupling to adenylate cyclase. Initial Northern blots of rat tissues indicated that the A_{2b} receptor was expressed by the central nervous system and, in a restricted manner, in other organs. While no expression was detected in rodent liver, kidney, small intestine, or heart, expression in rodent large intestine, urinary bladder, and lung was found (18). Adenosine is a known secretagogue in the ileum and colon (2, 3) and is the major direct-acting secretagogue released from stimulated neutrophils (8, 11), although it is likely that other PMN products might also contribute to Cl^- secretion through indirect actions mediated by subepithelial cells (40). Neutrophils release this secretagogue in the form of 5'-AMP, which is then converted by an ectoenzyme on the intestinal epithelial cell apical membrane to authentic adenosine (11). During states of active intestinal inflammation, activated neutrophils accumulate in the colonic crypts, the site of electrogenic chloride secretion (41), where the neutrophils have direct access to the apical membrane ectoenzyme as well as the apical AdoR (15, 42). Indeed, such "crypt abscesses" are the major histological criteria used in evaluating active inflammatory intestinal disease (7, 42). Since it appears that this apical receptor is of the A_{2b} subtype and since free access to this receptor is afforded from the colonic lumen, an attractive form of therapy for Cl^- secretion related to active inflammation might be the lumenal application of non-metabolizable A_{2b}-specific antagonists.

In summary, human intestinal epithelial cells and human colonic tissue contain abundant adenosine receptor of the A_{2b} subclass. Both the apical and basolateral receptors of T84 cells appear to represent A_{2b} receptors. The signaling pathway for both apical and basolateral adenosine-mediated Cl^- secretion is via cAMP. Lastly, basolateral stimulation with the natural agonist is less sensitive than that observed with apical agonist stimulation due to polarized uptake of authentic adenosine. Apical adenosine is known to stimulate intestinal secretion in natural as well as cultured intestinal epithelium, and adenosine is held to be a potentially key agonist associated with inflammatory cell infiltration of mucosa (43, 44). The expression of this unique subclass of adenosine receptor at this site opens the vista of lumenally directed, A_{2b} subclass-specific receptor antagonists for the treatment of secretory diarrhea associated with inflammation.

Acknowledgments—We gratefully acknowledge Dr. Ray Frizzell for valuable discussions and thank John Lee and Charlene Delp-Archer for expert technical assistance.

REFERENCES

1. Pratt, A. D., Clancy, G., and Welsh, M. J. (1986) *Am. J. Physiol.* 251, C167–C174
2. Dobbins, J. W., Laurenson, J. P., and Forrest, J. N., Jr. (1984) *J. Clin. Invest.* 74, 929–935
3. Grasl, M., and Turnheim, K. (1984) *J. Physiol. (Lond.)* 346, 93–110
4. Binder, H. J., and Sandle, G. I. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed) pp. 1389–1418, Raven Press, New York
5. Halm, D. R., and Frizzell, R. A. (1990) in *Textbook of Secretory Diarrhea* (Lebenthal, E., and Duffey, M., eds) pp. 47–58, Raven Press, New York
6. Weinstein, L., and Fields, B. N. (1978) *Seminars in Infectious Disease*, Vol. I, p. 89, Stratton Intercontinental Medical Book Corp., New York
7. Kumar, N. B., Nostrant, T. T., and Appelman, H. D. (1982) *Am. J. Surg. Pathol.* 6, 523–529
8. Madara, J. L., Parkos, C., Colgan, S., MacLeod, R. J., Nash, S., Matthews, J., Delp, C., and Lencer, W. (1992) *J. Clin. Invest.* 89, 1938–1944
9. Nash, S., Stafford, J., and Madara, J. L. (1987) *J. Clin. Invest.* 80, 1104–1113
10. Colgan, S. P., Matthews, J. B., Parkos, C. A., Delp, C., Awtrey, C. S., and Madara, J. L. (1992) *J. Leukocyte Biol.* 52, 183–187
11. Madara, J. L., Patapoff, T. W., Gillece-Castro, B., Colgan, S. P., Parkos, C. A., Delp, C., and Mrsny, R. J. (1993) *J. Clin. Invest.* 91, 2320–2325
12. Marquardt, D. L., Gruber, H. E., and Wasserman, S. I. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 6192–6196
13. Barrett, K. E. (1993) *Am. J. Physiol.* 265, C859–C868
14. Barrett, K. E., Huott, P. A., Shah, S. S., Dharmsathaphorn, K., and Wasserman, S. I. (1989) *Am. J. Physiol.* 256, C197–C203
15. Parkos, C. A., Delp, C., Arnaout, M. A., and Madara, J. L. (1991) *J. Clin. Invest.* 88, 1605–1612
16. Parkos, C. A., Colgan, S. P., Delp, C., Arnaout, M. A., and Madara, J. L. (1992) *J. Cell Biol.* 117, 757–764
17. Reppert, S. M., Weaver, D. R., Stehle, J. H., and Rivkees, S. A. (1991) *Mol. Endocrinol.* 5, 1037–1048
18. Stehle, J. H., Rivkees, S. A., Lee, J. J., Weaver, D. R., Deeds, J. D., and Reppert, S. M. (1992) *Mol. Endocrinol.* 6, 384–393
19. Rivkees, S. A., and Reppert, S. M. (1992) *Mol. Endocrinol.* 6, 1598–1604
20. Pierce, K. D., Furlong, T. J., Selbie, L. A., and Shine, J. (1992) *Biochem. Biophys. Res. Commun.* 187, 86–93
21. Londos, C., Cooper, D. M. F., and Wolff, J. (1980) *Proc. Natl. Acad. Sci. U. S. A.*

77, 2551–2554
22. Bruns, R. F., Lu, G. H., and Pugsley, T. A. (1986) *Mol. Pharmacol.* **29**, 331–346
23. Linden, J., Taylor, H. E., Robeva, A. S., Tucker, A. L., Stehle, J. H., Rivkees, S. A., Fink, J. S., and Reppert, S. M. (1993) *Mol. Pharmacol.* **44**, 524–532
24. Williams, M. (1987) *Annu. Rev. Pharmacol. Toxicol.* **27**, 315–345
25. Dho, S., Stewart, K., and Foskett, J. K. (1992) *Am. J. Physiol.* **262**, C67–C74
26. Barrett, K. E., Cohn, J. A., Huott, P. A., Wasserman, S. I., and Dharmsathaphorn, K. (1990) *Am. J. Physiol.* **258**, C902–C912
27. Madara, J. L., and Dharmsathaphorn, K. (1985) *J. Cell Biol.* **101**, 2124–2133
28. Shapiro, M., Matthews, J., Hecht, G., Delp, C., and Madara, J. L. (1991) *J. Clin. Invest.* **87**, 1903–1909
29. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
30. Mandel, K. G., Dharmsathaphorn, K., and McRoberts, J. A. (1986) *J. Biol. Chem.* **261**, 704–712
31. Welsh, M. J., Anderson, M. P., Rich, D. P., Berger, H. A., Denning, G. M., Ostedgaard, L. S., Sheppard, D. N., Cheng, S. H., Gregory, R. J., and Smith, A. E. (1992) *Neuron* **8**, 821–829
32. Halm, D. R., Rechkemmer, G. R., Schoucher, R. A., and Frizzell, R. A. (1988) *Am. J. Physiol.* **254**, C505–C511
33. Kemp, P. J., MacGregor, G. G., and Olver, R. E. (1993) *Am. J. Physiol.* **265**, L323–L329
34. Barrett, K. E., and Bigby, T. D. (1993) *Am. J. Physiol.* **264**, C446–C452
35. Cotecchia, S., Kobilka, B. K., Daniel, K. W., Nolan, R. D., Lapetina, E. Y., Caron, M. G., Lefkowitz, R. J., and Regan, J. W. (1990) *J. Biol. Chem.* **265**, 63–69
36. Bruner, G., and Murphy, S. (1993) *Neurosci. Lett.* **162**, 105–108
37. Mohuczy-Dominiak, D., and Garg, L. C. (1992) *Am. J. Physiol.* **263**, C1289–C1294
38. Wang, Y.-Z., Cooke, H. J., Su, H.-C., and Fertel, R. (1990) *Am. J. Physiol.* **258**, G432–G439
39. Nash, S., Parkos, C. A., Nusrat, A., Delp, C., and Madara, J. L. (1991) *J. Clin. Invest.* **87**, 1474–1477
40. Chang, E. B., and Rao, M. C. (1994) in *Physiology of the Gastrointestinal Tract* (Johnson, L. R., ed) pp. 2027–2081, Raven Press, New York
41. Welsh, M. J., Smith, P. L., Fromm, M., and Frizzell, R. A. (1982) *Science* **218**, 1219–1221
42. Fenoglio-Preiser, C., Lantz, P. E., Listrom, M. B., Davis, M., and Rilke, F. O. (1989) *Gastrointestinal Pathology: an Atlas and Text*, pp. 427–484, Raven Press, New York
43. Cronstein, B. N., Daguma, L., Nichols, D., Hutchinson, A. J., and Williams, M. (1990) *J. Clin. Invest.* **85**, 1150–1157
44. Cronstein, B. N., Levin, R. I., Philips, M., Hirschhorn, R., Abramson, S. B., and Weissman, G. (1992) *J. Immunol.* **148**, 2201–2206